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The biosystematics of pteridophytes:
Aspects of morphology and reproductive biology of some epiphytic ferns

by

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TABLE OF CONTENTS

ACKNOWLEDGMENTS iii

GENERAL INTRODUCTION 1

1. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF *CAMPYLONEURUM ANGUSTIFOLIUM* (SWARTZ) FEE 53

2. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF *CAMPYLONEURUM PHYLLITIDIS* (L.) PRESL 84

3. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF *LEPISORUS THUNBERGLANUS* (KAULF.) CHING 120

4. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF *MICROGRAMMA HETEROPHYLLA* (L.) WHERRY 151

5. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF *PHLEBODIUM AUREUM* (L.) J. SMITH 181

6. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF *PHYMATOSORUS SCOLOPENDRIA* (N. L. BURM.) PICI SERM. 207

7. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF *POLYPODIUM PELLUCIDUM* KAULF. 236

8. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY IN *ELAPHOGLOSSUM SCHOTT* 267

9. GENERAL CONCLUSION 299
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GENERAL INTRODUCTION

Although the usefulness of the gametophyte generation in phyletic comparisons among ferns was long neglected (Bower 1923; Holttum 1949), the significance of gametophytes in systematics has since been underscored (Nayar & Kaur 1971; Atkinson 1973). Because gametophytes are responsible for both habitat selection and sexual reproduction, analyses of the gametophytic generation must be included in fern biosystematic studies (Windham & Haufler 1986).

Research has shown that much of fern genetic variation, which is the crucial element of speciation and evolution, is related to the mating system expressed in the gametophyte generation (Lloyd 1974a; Klekowski 1979; Soltis & Soltis 1990). In some species, the only remaining generation of the life cycles is the independent gametophyte (Farrar 1985; 1990; 1992).

A number of studies have shown that gametophyte morphologies vary greatly beyond the standard heart shapes usually depicted in textbooks (Nayar & Kaur 1971; Dassler 1995). These variations include the ability to grow indeterminately and branch so that perennial gametophyte clones of considerable size may be produced. In some families, gametophytes also have the ability to reproduce themselves vegetatively by dispersable gemmae (Farrar 1971; Raine 1994; Dassler 1995). Gemma production and/or clonal growth have been observed primarily in epiphytic species (Dassler 1995). The significance of this morphological diversity to the evolution and systematics of ferns cannot be properly assessed without further knowledge of morphological variation and reproductive strategies of epiphytic species.

High genetic load, causing depression of sporophyte production, has been considered to be the primary mechanism promoting outcrossing and thus increasing genetic diversity (Haufler et al. 1990; Haufler and Welling 1994). However, genetic load of epiphytic ferns has been studied in very few species (C. Peck 1985; Raghavan 1989; Ranker 1992a).
Another factor indirectly affecting the mating system is the hormone (pheromone) antheridiogen, which stimulates antheridia formation and may induce fern spores to germinate in darkness (Naf 1979; Voeller 1971), both responses tending to promote outbreeding. Again, the antheridiogen system in epiphytic ferns has not been extensively investigated and most (the Polypodiaceae) are even thought to exhibit no response to antheridiogen (Voeller 1971; Raghavan 1989).

Most of the variation in gametophyte morphology is among epiphytic species whereas most of the information known on reproductive biology is from research on terrestrial ferns. Epiphytic ferns exist in very different environment from those of terrestrial species. The tropical habitat of gametophytes of epiphytic species is within dense bryophyte mats. In such habitats, interaction between gametophytes via chemical (antheridiogen) or sperm transfer must be significantly hindered relative to gametophytes of terrestrial ferns on smooth surfaces (Dassler 1995). Gametophyte morphology may play a crucial role in overcoming these difficulties to accomplish reproduction in the aerial habitat.

Most species of Polypodiaceae and Elaphoglossum (Elaphoglossaceae) are epiphytic, but their gametophytes are non-gemmiferous. I have chosen species of these two groups to observe their gametophytic morphologies and reproductive behavior. The results are expected to be helpful in understanding the systematics of these species and evolution as well as that of epiphytic species in general.

Dissertation organization

This dissertation is organized into a section of general introduction followed by nine chapters. The general introduction section includes an extensive literature review. Each following chapter is in the form of a journal article manuscript.

The first seven chapters are related to the morphology and reproductive biology of some Polypodiaceae gametophytes, i.e., Campyloneurum angustifolium (Swartz) Fee., C.
Phyllitidis (L.) Presl, Lepisorus thunbergianus (Kaulf.) Ching, Microgramma heterophylla (L.) Wherry, Phlebodium aureum (L.) J. Smith., Phymatosorus scolopendria (N. L. Burm.) Pichi Serm., and Polypodium pellucidum Kaulf. respectively. The eighth chapter is about the morphology and reproductive biology of several species in the genus Elaphoglossum (Elaphoglossaceae). Following the eighth chapter is the general conclusion giving a summary of this research.

Together these papers form my Ph.D dissertation.

**Literature review**

Pteridophytes, a group of vascular plants with two independent generations (the sporophyte and the gametophyte), include fern allies and ferns. The former are microphyllous whereas the latter are megaphyllous. There are two kinds of ferns: homosporous ferns and heterosporous ferns. Sporophytes of the latter produce two kinds of spores, mega- and microspores, which develop into female and male gametophytes, respectively. On the other hand, sporophytes of homosporous ferns yield only one kind of spores which potentially produce bisexual gametophytes. In this dissertation, only homosporous ferns are considered. Unless specifically noted, all mention of "ferns" below refers to homosporous ferns.

The life of a typical fern gametophyte starts with the mature spore, and after spore release and germination, continues with prothallus development and gametangium formation, and ends in successful fertilization producing the zygote and embryonic stages of sporophyte phase of the life cycle. This review includes all of the stages of the gametophyte phase of the life cycle.

**Spore Maturation and Release**

Few papers have described the phenology of spore maturation and release. The time of spore production may differ from that of release. For example, Matteuccia and Onoclea in temperate regions produce spores in the fall but release most spores the following spring.
which is the period of maximum rain (Hill and Wagner 1974; Wagner 1974; Wagner and Wagner 1977). Hill and Wagner (1974) also stated that the discharge season was related to the spore wall texture, sorus structure, and chlorophyll content. Farrar and Gooch (1975) observed the time of spore release, the period of maximum release, and spore viability at release time of 13 species at Woodman Hollow State Preserve in Iowa. Later, Farrar (1976) surveyed the proportion of unopened sporangia from December to March and found different spore retention ability among species. Peck (1980) also investigated the duration of maximum spore release and found that the period varied from 4 to 8 days (Osmunda claytoniana) to 3 to 4 months (Matteuccia struthiopteris), depending on species. However, the release rate may be influenced by the weather. For example, Peck (1980) compared the amount of spore retention in two years and concluded that wet years favor spore retention while dry years favor spore release. Peck (1980) also noted that the optimum time of spore release was at the time of maximum development of the forest canopy. Most papers mention spore collection dates (e.g., Stone 1969; Yoro 1972), but those dates might differ from the real spore availability in nature.

**Spore Productivity**

Similarly, spore productivity has not been extensively surveyed. Due to its economic impact, the earliest studied species is possibly Pteridium aquilinum, in which a frond might produce as much as one gram of spores (Schwabe 1951) or three hundred million spores (Conway 1957). Page (1979) observed twelve cultivated tropical and temperate ferns and estimated approximate spore numbers of from 750,000 (Asplenium trichomanes) to 750 million (Dicksonia antarctica) spores per frond, depending on the size of the frond and species. Conant (1976) estimated that Cyathea arborea could yield 30 billion spores annually on a leaf about 3 m long. Schneller (1979) estimated that Athyrium felix-femina could
produce 15 million spores on one frond and 11 million spores per square meter. Tryon (1986) estimated *Hecistopteris pumila* would produce 100,000 spores on a leaf about 2-3 cm long.

At Woodman Hollow State Preserve, Iowa, Farrar and Gooch (1975) and Farrar (1976) measured spore numbers on fronds, and estimated that spore numbers varied from fewer than 100,000 to more than one million on each frond. Peck (1980) counted the average number of fertile fronds per plant, average number of sporangia per fertile frond and the average number of spores per sporangium, and calculated the number of spores produced by each species at Woodman Hollow in one year. They estimated approximately 54,000 (*Cryptogramma stelleri*) to 325 million (*Dryopteris goldiana*) spores per plant. They also estimated that the annual spore production by species in Woodman Hollow, 26 hectares, ranges from 16 million (*Cryptogramma stelleri*) to 600 billion (*Woodsia obtusa*), and that annual spore releases varied from 1.6 million (*Cryptogramma stelleri*) to 540 billion (*Woodsia obtusa*).

**Spore Dispersal**

After spores mature on fronds, the dehiscence mechanism of the annulus propels spores into the air where they may be transported by air currents. Once into the air, spore transportation will be affected by physical factors such as wind currents, gravity, temperature, rainfall, electrostatic and photophoretic forces, sending spores to long distances (Gregory 1945). Pollen grains, fungus spores, and some undetermined spores have been trapped at heights of 5000 to 7500 feet (Stakman *et al.* 1923). Rust spores have even been trapped at a height of 16,500 feet (Ingold 1939).

Most discharged spores tend to be trapped by surrounding vegetation (Janssen 1972; Tauber 1967). Thus it is more difficult for spores from forest species to disperse to distant suitable sites (Whitehead 1969). Magraw and Musselman (1979) trapped most *Dryopteris* spp. spores at ground level and fewer at 1.5 m above ground level and at forest canopy level,
and presumed that this was due to the relatively mild air currents within the dense forest. In the forest of Woodman Hollow, Peck (1980) monitored and mapped the dispersal of *Botrychium virginianum* spores with spore traps and found that the higher the sporophore was, the farther the spores were transported. This was particularly evident when the sporophore extended above the herbaceous canopy. In all cases known, the great majority of spores were deposited within a few meters of the sources. Generally, spore dispersal follows a leptokurtic pattern (Ingold 1939).

Spore dispersal is also related to spore wall structure. Kramer (1977) proposed a synaptospory hypothesis in which perispore sculpture tends to stick spores together into a dispersal unit and thus promotes intergametophytic selfing. In *Dryopteris* spp., Schneller (1975) showed that some spores were shed in groups of two, three, and four or more. On the other hand, fern spores of most epiphytic genera, with a few exceptions such as *Elaphoglossum*, lack perispores, and hence a single spore dispersal is favored (Kramer 1977).

Different kinds of multispore transport have also been mentioned. Klekowski (1979) noted that entire sporangia of *Matteucia struthiopteris* shed during winter and were transported by snowmelt in the spring. Gastony (1974) found that the sporangia of some tree ferns dispersed with many spores remaining inside. In *Asplenium lepidum*, Brownsey (1977) observed that the majority of sporangia retained their full spore content and did not open even when the frond dried out. Although there is no direct observation, C. J. Peck (1985) mentioned that catastrophic storms, hurricanes, typhoons, or tornados might transport intact sporangia, frond fragments, or even entire plants over a long distance. All these multispore transports may cause subsequent gametophytes to grow in close proximity, thus promoting intergametophytic mating.

Airborne spores are possibly carried for long distances before they land. Direct evidence of long distance dispersal of fern spores has been recorded. Polunin (1951) trapped
ferns in the arctic by aircraft. Using radioactive phosphorous, Conant (1978) labeled spores of *Cyathea arborea* and detected that spores flew at least 30 meters. Other indirect evidence of long distance dispersal has been provided from studies of bryophyte spores (Wyatt 1982), fungi spores (Newman 1948; Burchill 1966), dust and smoke particles (Bull 1951), and air pollutants (Reed 1976; Ottar 1976; Lunde 1976).

Historical records provide some evidence of long distance dispersal of fern spores. After the catastrophic volcano eruption at Krakatoa Island, ferns have been thought to spread to new habitats through long distance dispersal of spores (Campbell 1909). Some fern species have been found after pine and spruce were planted about 50 years ago near Ann Arbor, Michigan, about 200 miles away from the nearest locality for these species (Wagner 1972).

Geographical data have also provided evidence of long-distance dispersal of fern spores. Tryon (1970; 1972) studied fern geography of ocean islands and tropical America and found that many ferns were disjunct up to 500 miles away from other sources. Wagner (1972) found ferns to be abundant in the metropolis regions (= source point) and relatively scarce in disjunct outlier areas which were possibly 2000 miles away from the source point. Based on his observation and other evidence (e.g., Klekowski and Baker 1966; Klekowski 1972a) indicating that ferns are capable of intragametophytic selfing, Wagner (1972) believed that "a single spore can start a colony". Crist and Farrar (1983) showed that *Asplenium platyneuron* on isolated coal spoil areas in Iowa have been established through long-distance dispersal and successful intragametophytic selfing due to low genetic load. Wide disjunctions have also been found in some species of *Botrychium* (Wagner and Wagner 1994), which also tend to reproduce by intragametophytic selfing (McCausley *et al.* 1985; Soltis and Soltis 1986). However, some disjunct outlier populations may be remnants or relicts of old distribution, e.g., in the Driftless Area of Northeast Iowa and Southwest Wisconsin, rather than from long-distance dispersal (Peck 1982). Long-distance dispersal of spores has also been indicated by
spore banks (see below) which lack sporophytes growing nearby (Milberg and Anderson 1994).

Despite the abundance of examples of establishment by long-distance dispersal, there is strong evidence that species are not equivalent in their ability to migrate by long-distance dispersal. Wagner (1972) and others have pointed out that spore-bearing plants tend to have the same occurrence patterns as seed plants. Furthermore, environmentally diverse but geographically distant islands (e.g., Hawaii) have only a small fraction of the species present in potential source areas (Dassler 1995). It must be pointed out also that spore dispersal does not necessarily result in species establishment. Reasons for this include various physiological and ecological requirements for spore viability and germination, gametophyte and sporophyte growth, reproductive behavior, etc. (Tryon 1986; Peck et al. 1990; Werth and Cousens 1990; and this review in subsequent sections).

Besides horizontal spore dispersal, vertical transportation in soil has also been studied. Dyer and Lindsay (1992) have reviewed in detail the depth to which fern spores have been found in different habitats and locations. A spore bank in an old deciduous woodland near Edinburgh, Scotland, revealed viable spores below the surface down to 125 cm (Dyer and Lindsay 1992). A general trend is the reduction of spore bank size with depth (Wee 1974; Leck and Simpson 1987; Hamilton 1988; Schneller 1988). How fast (or slowly) the spores penetrate below the surface layer is not clear. The downward spore movement seems most likely to be caused by rain water percolating through the substrate (Schneller 1988). Animal activity may also contribute to the downward movement of spores. Earthworm activity has been related to spore transportation in the spore bank (Schneller 1988; Hamilton 1988). Spores may be washed through worm channels, carried by worms into deep soil, and passed through the earthworm gut without being damaged (Schneller 1988; Hamilton 1988). The last point has been proven by Van Tooren and During (1988) who extracted viable
spores from earthworm guts. Other invertebrate and vertebrate soil fauna also play possible roles in the redistribution of spores, and are capable of carrying the buried spores upward to safe germination sites (Dyer and Lindsay 1992; Hamilton 1988). Erosion, disturbance, and turning over of soil may bring spores up to the soil surface as well (Schneller 1988).

**Spore Viability**

Spore viability varies among fern species. The spores of *Pellaea* stored in the herbarium can remain viable for nearly 50 years (Windham *et al.* 1986). The fern spores with the longest viability have been found in a heterosporous fern, *Marsilea oligospora*, of which spores survived for 100 years (Johnson 1985). However, the life span of most fern spores is usually more limited (Miller 1968). Fern spore viability was first reviewed in detail by Lloyd and Klekowski (1970) who classified spores into two groups, *i.e.*, green and non-green spores, and generalized that green spores germinate faster and have less viability lengths than non-green spores. However, Dassler (1995) demonstrated that green spores of epiphytic ferns remained viable and relatively ungerminated within a bryophyte mat while non-green spores of two species (*Polypodium pellucidum*, *Pityrogramma austroamericana*) and green spores of a terrestrial species (*Matteuccia struthiopteris*) germinated rapidly and became etiolated under the same conditions.

Generally, the germination of spores declines and become delayed as the storage time increases. For example, the germination rate decreased for aged spores of *Polypodium vulgare* and showed a sigmoid curve (Smith and Robinson 1975). Beri and Bir (1993) found that germination and the contents of sugar, amino acids, as well as protein gradually declined in spores of *Pteris vittata* after being stored 20 days in room temperature, and thus concluded that the reduced germination of spores was associated with their biochemical loss.

Fern spores, traditionally, have been stored in dry and cold conditions. Recently, fern spores stored in wet conditions have been studied. Lindsay *et al.* (1992) sowed non-green
spores of five species on agar, stored them in the dark at 20°C for two years, and found that
the germination time was earlier and germination rate was higher than for spores stored in
dry conditions. Similarly, Whittier (1990) showed that spores of *Psilotum nudum* survived
longer when stored on mineral agar. The reason may be due to the continuous replacement or
neutralization of old and damaged cellular products, thus slowing down the process of aging
as in the seeds of some seed plants (Lindsay *et al.* 1992; Villiers 1974; Villiers and
Edgecumbe 1975).

In nature, spores on fronds and during transportation periods are usually exposed to
the environment more varied and severe than spores that are stored artificially. Most fern
spores can withstand, to some extend, UV, X-ray, Gamma radiations, and cold temperatures
(Charlton 1938; Knudson 1940; Miller 1968). Farrar and Gooch (1975), Farrar (1976), and
Schneller (1979) detected the viable spores remaining in sporangia on fronds in the following
spring after spore maturation. Spore viability in spore banks has been found to be at least
over one year (Schneller 1979; 1988) or two years (Dyer and Lindsay 1992).

Types of Spore Germination

Several spore germination types have been described for ferns. Based on the relative
direction of the first protonema cell and the first rhizoid, Momose (1942) classified spore
germination into three types: centrifugal, tangential, and centripetal. Atkinson and Stokey
(1964) reported five germination types but did not name them except for bipolar and tripolar
types. After observing more species, Nishida (1965) modified some types and added others.
Nayar and Kaur (1968; 1971) argued that earlier researchers had ignored the polarity of
spores. They thus classified the germination type as Amorphous, Polar, and Equatorial types
based on the plane of the first cell division relative to the tetrad attachment site. The
amorphous type is restricted to some relatively primitive groups. The polar type includes
Anemia-, Osmunda-, and Vittaria-types. The equatorial type includes Gleichenia-,
Christiopteris-, Cyathea-, Hymenophyllum-, Trichomanes-, and Mecodium-type of which the last three are characteristic of Hymenophyllaceae. Recently, however, Raghavan (1989), thought previous observations were based on "whole mount" preparation of materials and thus questioned those results. He therefore reclassified the types, based on "section" observation, as 'Ceratopteris', 'Lygodium', 'Vittaria', 'Osmunda', 'Dicksonia', 'Gleichenia', 'Hymenophyllum', 'Mecodium', and 'Trichomanes' types.

Factors of Spore Germination

Light  Fern spore germination is strongly affected by light (Miller 1968; Raghavan 1989; 1992). As in seed germination, the $P_{r}$-$P_{f}$ phytochrome system controls fern spore germination, i.e., red light promotes germination while far-red light inhibits it (Raghavan 1971; 1992; Haupt and Filler 1986). The pigment and its phototransformation has been demonstrated in the spores of *Lygodium japonicum* (Tomizawa et al. 1982). Phytochrome may be synthesized during sporogenesis or possibly upon hydration of the spores (Schraudolf 1987; Manabe et al. 1987). Besides far-red light, blue light and UV-light also inhibit spore germination, and both effects can be reversed by red light. Thus the blue and near UV-light absorbing pigment (Pb-nuv) is judged to participate in the photoreaction (Sugai 1971; Sugai et al. 1984). Raghavan (1992) presumed that the phytochrome interacts with the blue-light-sensitive pigment and functions to inhibit spore germination. However, the degree of sensitivity to those lights of various wavelength are different among species such as *Adiantum capillus-veneris*, *Ceratopteris richardii*, and *Onoclea sensibilis* (Cooke et al. 1995). In addition, Ca$^{2+}$ may act in the phytochrome-mediated function, at least in sensitive fern, because in *Onoclea*, germination occurred only if both red light and a calcium-rich medium were furnished (Wayne and Hepler 1984), and because the red light stimulated an increase of internal calcium in the spores of *Onoclea* (Wayne and Hepler 1985).
Besides light quality, light intensity and duration also affect germination. In general, it takes more time at lower light intensity to germinate than at higher intensity (Raghavan 1989). However, the most appropriate intensity is dependant on the species, and this may relate to adaption to the habitat (Hill 1971). The influence of light intensity may begin in sporogenesis. For example, in *Polypodium aureum*, only 24% of the spores collected during October-January germinate whereas 79% of spores collected during January-April germinate. This higher germination rate has been attributed to the prolonged exposure to light during spore maturation (Spiess and Krouk 1977).

Except for a few species which can germinate spontaneously in darkness, the germination of most fern spores is light-dependent (Miller 1968). However, some species can be induced to germinate in darkness by different treatments, e.g., age, higher temperature, nutrient, hormone, and so on.

Some species germinate in darkness only when they are fresh. For example, fresh spores of *Onoclea sensibilis* are capable of germinating in darkness at about 20%, whereas aged spores (ca. two months) can germinate only under light (Miller 1968; Rubin *et al.* 1984). Similarly, *Polypodium hirsutissimum* germinated at 6.3% in darkness after 47-day storage, whereas germination failed after 90-day storage (Ranal 1991). Spores of *Osmunda regalis* lost the ability to germinate in darkness after two months but still germinated under light after five months (Laage 1907, cited from Miller 1968).

Germination in darkness may be induced by higher temperatures for some light-dependent spores. Spores of *Alsophila loddigesii* were induced by raising the temperature to 32°C (Heald 1898), *Ceratopteris thalictroides* by raising the temperature to 32-35°C (Heald 1898; Miller 1968), *Gymnogramme calomelanos* by raising the temperature to 30-33°C (Life 1907), and *Onoclea sensibilis* by elevating temperature to 28-29°C (Hartt 1925).
Adding nutrient to the medium also promotes spore germination in darkness. This has been studied in *Ceratopteris thalictroides* (Nagai 1914, cited from Miller 1968), *Dryopteris spinulosa* (Laage 1907, cited from Miller 1968), *Osmunda regalis* (Laage 1907 and Stephan 1929, cited from Miller 1968), and so on.

Gibberellic acid substitution for the light requirement for spore germination has been demonstrated in the family Schizaeaceae, *i.e.*, *Anemia, Lygodium, Mohria* (Naf 1966; Weinberg and Voeller 1969a, b; Nester and Coolbaugh 1986). However, neither *Schizaea pusilla*, a member of Schizaeaceae (Guiragossian and Koning 1986), nor other tested families responded to this hormone (Weinberg and Voeller 1969a).

Antheridiogen (or germinin, Voeller 1971), a natural hormone produced from mature gametophytes, is able to substitute for the light requirement and induce spore germination in darkness for some species which have been listed by Weinberg and Voeller (1969a). Gametophytes of a number of species have been shown to produce antheridiogen and promote their spores to germinate in darkness, *e.g.*, *Anemia mexicana* (Nester and Schedlbauer 1982), *Pityrogramma calomelanos* (Dubey and Roy 1985), *Pteris vittata* (Gemmrich 1986), *Athyrium filix-femina* (Schneller 1979), *Dryopteris filix-mas, D. affinis* (Schneller 1988), *Polypodium australe* (Welling and Hafler 1993), *Bommeria ehrenbergiana*, and *B. hispida* (Hafler and Welling 1994).

Antheridiogen can penetrate soil down to at least 1 cm (down to 15 cm by Schraudolf's observation, stated by Schneller 1988) and stimulate buried spores to germinate and promote antheridium formation on the subsequent gametophytes (Schneller 1988). Since light enters at most into the upper 4 mm of the typical forest soil (Bliss and Smith 1985; Kasperbauer and Hunt 1988), antheridiogen secreted by the larger mature gametophytes may play an important role in provoking spore-bank spores into germinating and forming
available sperms for outcrossed fertilization (Voeller 1971; Schneller et al. 1990; Haufler and Welling 1994).

Although light promotes most spores to germinate, light inhibition of germination occurs in some subterranean gametophytes. For example, all the species studied in the family Ophioglossaceae, i.e., Botrychium, Ophioglossum, and Helminthostachys, germinate in darkness, but fail to do so when their spores are exposed under light (Whittier 1973, 1981; 1987; Gifford and Brandon 1978).

**Temperature** Generally, most species are able to germinate in the range of 15°C-30°C, whereas the optimum range for most species is 20-25°C (Miller 1968; Raghavan 1989). This variation has been attributed to adaptation to the environment (Hill 1971). For example, the optimum germination temperature of some xeric species of Notholaena, Pellaea, and Cheilanthes varied from 20°C to 35°C and spores of these species tolerated temperatures up to 40-50°C and down to 0-5°C. The more xeric species are more tolerant of high temperatures and less tolerant of low temperatures than those less xeric species (Hevly 1963). Comparing two tropical ferns (Ceratopteris thalictroides and C. pteridoides) and a temperate species (*Matteucia struthiopteris*), Warne and Lloyd (1980) observed that the spores of *Ceratopteris* could not germinate below 12°C, whereas *Matteucia* germinated through 11°C to 27°C. Hickok et al. (1995) found that temperatures lower than 26.5°C significantly delayed the spore germination in *Ceratopteris* which is of tropical origin.

**Water** Spores germinate after they absorb enough water. Both agar-solidified and liquid media are satisfactory for germination, but the latter is more favorable than the former (Raghavan 1989). Hurel-Py in 1944 observed that seven species germinated better in liquid media than in agar media (cited in Miller 1968). This is possibly because entry of water into the spore is determined by the thickness of spore wall, the breakage of which is hindered in the solid medium (Raghavan 1989).
In general, the optimum pH range for germination is slightly acid to neutral (Miller 1968; Raghavan 1989). For example, *Pteridium aquilinum* has an optimum range of 5.5-7.5 (Conway 1949), and *Gymnogramme sulphurea* has an optimum range of 4.7-7.0 (Guervin and Laroche 1967). The various pH requirements seem related to the habitats. For instance, Hevly (1963) found that limestone ferns withstand higher pH level to pH 8-10, while igneous species tolerate lower pH levels (pH 5-6).

**Sterilization** Diluted calcium hypochlorite, sodium hypochlorite or clorox have been widely used to eliminate contamination by microorganisms for gametophyte cultures (Dyer 1979). However, surface sterilization might reduce spore germination rates. For example, Howland and Boyd (1974) found germination reduced as much as 50% after sterilization with 5% Clorox in spores of *Pteridium aquilinum*. Sterilization could either remove the perispore (Marengo 1956) or inhibit cell wall growth (Crotty 1967). On the other hand, little or no reduction of spore viability through sterilization has also been found in some species (Knudson 1940; Ward 1954; Dassler 1995).

Several improved methods of spore sterilization have been used. A prior soaking with a wetting agent decreases the required sterilizing time (e.g., Naf 1958; Bell 1961). Another effective way is to sterilize after imbibing spores in the dark through an appropriate period (ca. 24-36 hours) during which fungi germinate and bacteria grow (Voeller 1964; Haufler and Gastony 1978).

**Fungi** The presence of fungi may enhance spore germination unless the gametophytes are overgrown by fungi (Miller 1968). For example, Bell (1958) inoculated unidentified fungi onto mineral media and observed increased germination of spores in *Thelypteris palustris, Polystichum lobatum*, and *Dryopteris filix-mas*. In *Pteridium aquilinum*, spores in cultures containing fungi germinated better than those in pure cultures (Hutchinson and Fahim 1958). However, a claim that fungi can inhibit fern spore
germination, because fungi colonies produce inhibitors (aldehydes, ethanol, methanol, etc.) of fungal spore germination (Robison and Park 1966), has been made (Page 1979).

Allelopathy Research on fern allelopathy is very limited, and most of it concerns the effects of bracken fern on other weeds or crops (Rice 1984). Some allelopathic studies that considered the effects of both gametophyte and sporophyte on spore germination have been studied. Bell (1958) found that the prothallial debris of *Dryopteris filix-mas* inhibited spore germination in *D. borreri*, but the aqueous extract of the former promoted spore germination as well as gametophyte growth of the latter. Munther and Fairbrothers (1980) noted that the extracts of leaves of *Osmunda claytoniana*, *O. cinnamomea*, and *Dennstaedtia punctilobula* significantly inhibited their spore germination, and the latter two species extract also inhibited germination of the other two species. Star and Weber (1978) further observed that sporophytic extract of *Pityrogramma* inhibited germination of their spores. However, Peck (1980) reported that spore germination was not affected by frond fragments of sporophytes or by the substrates at the base of sporophytes in Woodman Hollow, Iowa, and suggested that compounds leached from sporophytes do not directly control spore germination.

Research into the effects of spore density on germination has also shown the presence of allelopathy. For example, in *Polypodium vulgare*, at lower levels of density, e.g., 0.01-30 spores/mm², spore germination increased with increasing density. On the other hand, at extremely high densities, e.g., $10^3$-$10^6$ spores/ml, the greater the density of the spores, the greater germination was inhibited (Smith and Rogan 1970; Smith and Robinson 1971). Similar results have been observed in *Matteucia struthiopteris* (Pierykowska 1962a). Although this was attributed to insufficient light intensity in the high density cultures (Pierykowska 1962a), Smith and Robinson (1971) argued that intensity might be significantly diminished at the later developmental stage but not at the early germination
period. Smith and Robinson (1971) also detected (but did not identify) three stable factors produced from germinating spores, and presumed that these factors inhibited spore germination in very high concentrations but promoted germination at relatively low concentrations. Thus they interpreted inhibited germination of spores in high density as possibly due to the high accumulation of these factors, while the slower and delayed germination at very low density is due to these factors being below the promotive level.

**Gametophyte Morphology and Development Types**

The morphological characteristics of many species of fern gametophytes were first described by Stokey (1951). After more observations, Atkinson and Stokey (1964) published detailed reviews of gametophyte morphology and development types and concluded that the gametophyte could shed light on the phylogenetic relationships of ferns. Later, Atkinson (1973) illustrated gametophyte characteristics of each family or genus. Another elaborate review paper was published by Nayar and Kaur (1971) who classified spore germination types, gametophyte morphologies, as well as gametophyte development types, and outlined gametophyte characteristics in all families. They further constructed a phylogenetic cladogram based on the features of spores and gametophytes.

In addition to the typical heart or cordate shapes in most fern gametophytes, other types have also been described (Reviewed in Nayar and Kaur 1971; Dassler 1995). For example, unbranched to branched strap-like forms with cordate apices and ribbon-like forms with rounded apices develop in some families: Grammitidaceae, Polypodiaceae, Lomariopsidaceae, Hymenophyllaceae and Vittariaceae. The filamentous type also occurs in Hymenophyllaceae and Schizaeaceae. In Vittariaceae, Hymenophyllaceae and Grammitidaceae, special vegetative propagules, gemmae, are able to promote local distribution and indefinite maintenance of populations (clones) (Farrar 1971; Raine 1994; Dassler 1995).
Even in typical cordate-shaped gametophytes, branching may occur in some situations, especially under prolonged cultures (Mottier 1925; 1927; Walp and Proctor 1944; Wuist 1916). Proliferations are able to develop not only on nutrient media, but also on distilled water and on the soil (Wuist 1916; Cousens 1979). Proliferations may also be produced from either the margin or dorsal or ventral surface of the parent gametophyte (Atkinson and Stokey 1964). Proliferations may occur spontaneously or be induced by weak light, starvation, crowding, or injury (Albaum 1938; Walp and Proctor 1944; Raghavan 1989).

Subterranean gametophytes are found in Ophioglossaceae and Schizaeaceae. The former are tuberous and obligately subterranean. The latter include Schizaea, Lophidium, and Actinostachys and their gametophytes vary from unbranched to branched, uniseriate to multiseriate, filamentous to tuberous and either subterranean or surface-living (Nayar and Kaur 1971; Dassler 1995).

Gametophyte Development Factors

Light  Besides controlling spore germination, light affects gametophyte development: continuously grown under very low light intensity, gametophytes remain filamentous (Conway 1949), whereas strong light shifts filamentous growth to planar growth (Miller and Miller 1961). Two light qualities affect cell divisions. Basically, irradiation of red light retains gametophytes in the filamentous phase whereas blue light increases cell division and inhibits cell elongation (Raghavan 1989). Examples of this control have been demonstrated in several species, e.g., Pteridium aquilinum (Davis 1968), Pteris vittata (Kato 1965), Phymatodes nigrescens (Yeoh and Raghavan 1966), and so on. However, the sensitivity of gametophyte growth to light intensity varies, depending on the species (Raghavan 1989; Cooke et al. 1995).
In addition to influencing gametophyte growth, blue light also promotes antheridium development whereas red light inhibits antheridium development (Grill 1988). Ca\(^{2+}\) considerably enhances antheridium formation in blue light (but no effect in red light) for *Anemia phyllitidis* (Grill 1995). Conversely, the antheridium development of *Pteris vittata* was inhibited in blue light when Ca\(^{2+}\) was applied (Gemmrich 1988). The reason for these opposite results is unclear.

One aspect of light that has rarely been mentioned is the influence of light position. Illumination from the side or below the substrate might affect the establishment of dorsiventrality and patterns of development (Bussman 1939, cited from Miller 1968; Wada and Furuya 1971). In artificial cultures, especially on agar or liquid cultures using transparent containers, light enters not only from above but also from the sides and from below. Dyer (1979) suggested using black culture dishes to avoid lateral and basal light.

**Temperature** Various temperatures have been widely used in gametophyte cultures in which the range of 18\(^{\circ}\)C-28\(^{\circ}\)C was most frequent (Dyer 1979). In general, every species has its optimum temperature range for growth. For example, Hill (1971) noted that the optimum range was 15-25\(^{\circ}\)C for *Adiantum pedatum*, 25-30\(^{\circ}\)C for *Thelypteris palustris*, and 25\(^{\circ}\)C for *Woodwardia virginica*. Inappropriate temperature may affect gametophyte morphology. Kawasaki (1954) reported that the gametophytes of *Cyathea bonensimensis* was cordate at 20\(^{\circ}\)C-30\(^{\circ}\)C but remained filamentous at 5\(^{\circ}\)C-10\(^{\circ}\)C.

Temperature may interact with other environmental conditions in affecting growth. Dyer and King (1979) observed that division of prothallial cells was fastest at 20\(^{\circ}\)C at lower light intensity, and at 22.5\(^{\circ}\)C or a little above at higher intensity in *Dryopteris pseudo-mas*. In *Onoclea sensibilis*, filaments increased their length the most between 20\(^{\circ}\)C and 22\(^{\circ}\)C under darkness or red or far-red light illumination, and the filament length increased steadily between 20-29\(^{\circ}\)C when treated by blue light (Miller and Miller 1966).
Gametophytes are capable of resisting very severe cold temperatures. Farrar (1978) reported that the independent Appalachian *Vittaria* gametophytes could survive freezing at $-10^\circ C$. More than 80 temperate species have been observed and all of those gametophytes, with very few exceptions, are hardier than sporophytes and can resist temperatures below $-5^\circ C$, (in extreme species down to $-70^\circ C$), and regrow into normal plants when they are relocated to room temperature (Sato and Sakai 1980; 1981; Sato 1982).

**pH** Although the optimum pH for spore germination has been extensively tested, very few papers have concentrated on pH effects on gametophyte growth. Hill (1971) reported that gametophytes grew well at pH 5 to pH 8 for *Adiantum pedatum*, at pH 5-7 for *Woodwardia virginica*, and at pH 5 for *Thelypteris palustris*, but all of them survived between the tested range, *i.e.*, pH 4-10. Kiss *et al.* (1995) noted that the optimum pH was 4.5 for the gametophyte growth of *Schizaea pusilla*, a species which grows in acid bogs.

**Substrate** Gametophytes have been successfully cultured on a variety of solid and liquid media (Miller 1968). The latter is convenient for some specific purposes, *e.g.*, antheridiogen preparation (Naf 1956), but normal growth is limited to gametophytes floating on the surface while those sunken are usually filamentous, possibly due to oxygen deficiency (Miller 1968). Most research uses solidified agar media which may cause gametophytes to proliferate more than when on soil. Peck (1980) reported that gametophytes grown in nature were relatively less proliferated than those grown on agar.

**Sterilization** Sterilization may or may not affect gametophyte development or morphology. Proliferations and abnormal developments are likely to occur in sterilized media. Steeves *et al.* (1955) cultured *Pteridium aquilinum* in sterile cultures and classified those irregular gametophytes into five types: the filamentous proliferation, the pincushion proliferation, the coralloid proliferation, filamentous pseudocallus, and parenchymatous callus. Crotty (1967) noted that cell wall growth was inhibited by sterilant in *Pteris vittata*. In
Onoclea sensibilis, Kotenko (1983) observed that dilute calcium hypochlorite reduced cell division, although there was no significant influence on morphology and antheridium formation. On the other hand, Smith and Rogan (1970) used 5% solution of calcium hypochlorite and found no difference in gametophyte growth between sterilized and unsterilized spores of Polypodium vulgare.

Gametophyte transfer  Isolated-gametophyte cultures have been used to study genetic load and mating systems (e.g., Lloyd 1974a; Klekowski 1979; Masuyama 1979). However, gametophyte transfer may affect development. For example, Kotenko (1983) observed that growth of transferred gametophytes was slower than those of nontransferred gametophytes. Also, prothallial colonies were more easily derived from small transplanted gametophytes than from large ones (Steeves et al. 1955). C. J. Peck (1985) described Farrar's personal observation that development disruption was likely to occur in Adiantum pedatum when young gametophytes were transferred rather than older ones, possibly because young gametophytes could not easily be transferred in their original orientation without disturbing their polarities.

Density  The fact that crowded populations of gametophytes cause abnormal development and morphology is well known. The common phenomena of gametophytes in high density conditions include a decrease of cell numbers, increase of gametophyte lengths, and delays in the transition from one dimensional to two dimensional growth (Steeves et al. 1955; Smith and Rogan 1970). Pietrykowska (1962b) even found that after several weeks of crowded conditions, gametophytes of Matteuccia struthiopteris had retained filamentous growth and were unable to resume normal growth when transferred to an uncrowded conditions. However, a contrary observation in Polypodium vulgare was noted by Smith and Rogan (1970), who indicated that the inhibition was not permanent and would be removed when gametophytes were shifted to a favorable condition.
High-density populations also favor the onset of small, male gametophytes, and this effect has been attributed to antheridiogen function (e.g., Schedlbauer and Klekowski 1972; Cousens 1975, 1979; Warne and Lloyd 1987).

Extremely low density growth has been performed in monospore or monogametophyte cultures which usually have been applied to research on genetic load (e.g., Schedlbauer and Klekowski 1972; Lloyd 1974a; Cousens 1979). However, in many cases, isolated cultures showed lower rates of germination, abnormal or slow gametophytic growth, and low survivorship (Bell 1958; Nester and Schedbauer 1982). This may be caused by insufficient accumulation of growth promoters secreted by gametophytes (Smith and Robinson 1971). Other biotic factors may also influence the morphology. For example, male gametophytes do not always appear in monospore or monogametophyte cultures if an antheridiogen system exists (e.g., Haufler and Gastony 1978), but bisexual gametophytes may occur in pioneer species to favor intragametophytic selfing (Lloyd 1974b).

**Fungi**

Fungi may either promote or delay prothallia development. Bell (1958) noted that the gametophytes of *Thelypteris palustris, Polystichum lobatum, and Dryopteris filix-mas* grew faster in fungi-inoculated media than those in non-inoculated media. On the other hand, although volatile metabolites from 14 species of fungi stimulated gametophyte growth in *Pteridium aquilinum*, two fungal species inhibited fern gametophyte growth (Hutchinson 1967). Smith and Robinson (1975) reported that two volatile substance, *i.e.*, ethanol and acetaldehyde, produced by some fungi, especially by *Fusarium oxysporum*, delayed the transition from one dimension to two dimensions in the gametophytes of *Polypodium vulgare*.

Some fern gametophytes, especially subterranean gametophytes, are associated with endophytic fungi, *e.g.*, *Botrychium* (Schmid and Oberwinkler 1994), *Helminthostachys* (Whittier 1987), and *Schizaea* (Bower 1923). For above-ground gametophytes, endophytic
fungi are found in relatively primitive groups, e.g., some Marattiaceae, Gleicheniaceae, and Osmunda (Campbell 1908; Schmid and Oberwinkler 1995) and Hymenophyllaceae (Farrar and Wagner 1968).

**Allelopathy**  Allelopathy between gametophytes has been reported in a very limited number of papers. Bell (1958) found that gametophytes of Dryopteris borreri were retarded when they grew on the media which included gametophytes of Dryopteris filix-mas. Petersen and Fairbrothers (1980) demonstrated that the gametophyte surpermatant of liquid cultures of Osmunda cinnamomea and Dryopteris intermedia inhibited each other's gametophyte growth.

Allelopathy between sporophytes and gametophytes has also been shown. Star and Weber (1978) observed that extracts of sporophytes inhibited gametophyte development in Pitroyrogramma calomelanos. Munther and Fairbrothers (1980) reported that the extracts of leaves of Osmunda claytoniana, O. cinnamonea, and Dennstaedtia punctilobula inhibited their gametophyte development, and except for O. claytoniana, extracts of the other two species also inhibited the development of each other. However, Peck (1980) tested 13 species in Woodman Hollow, Iowa, and found that all gametophytes grew to plate stages without influence by the media of sporophyte frond fragments or by the substrate at the base of sporophytes.

Two allelopathic compounds, produced from roots of Thelypteris normalis, have been studied, i.e., thelypterin A and B (Davidonis and Ruddat 1973). Lower concentration of these compounds retarded the formation of meristems whereas the higher concentration of the compounds arrested the gametophytes at 1-2 cell stages. However, growth resumed when the thelypterins were removed (Davidonis and Ruddat 1973; 1974). Thelypterin retarded not only the growth of gametophytes of T. normalis but also that of Pteris longifolia and Phlebodium aureum (Davidonis and Ruddat 1973; 1974). Other species have been found to possess
thelypterin A and/or B, e.g., thelypterin A and B in roots of *T. dentata*, thelypterin A in leaves of *T. noveborcensis*, and thelypterin B in roots of *Pteris vittata* and *P. multifidia* (Davidonis 1976).

**Antheridiogen**

Antheridiogen is a hormone (or pheromone) which is secreted by mature gametophytes and it promotes antheridium formation in other young gametophytes. The discovery, history, characteristics, and function of antheridiogen have been described in great detail by many authors, e.g., Miller (1968); Voeller and Weinberg (1969); Naf et al. (1975); Naf (1979) and Raghavan (1989). The tested species responsiveness to antheridiogen are also listed in the above reviewed papers and in their references. Other species producing antheridiogen have also been found, e.g., *Dryopteris affinis* (Schneller 1988), *Phanerophlebia nobilis*, *P. unbonata*, *Cyrtomium fortunei*, *C. macrophyllum*, *Polystichum acrostichoides* (Yatskievych 1993), *Polypodium australis* (Welling and Haufler 1993) and *Bommeria palmata* (Schneller et al. 1990). All of these species were assayed in the laboratory. Antheridiogen effects have rarely been observed in the field, due to the troublesome identification of gametophyte species. This problem is particularly acute when several species with similar morphology grow together (Tryon and Vitale 1977; Hamilton and Lloyd 1991).

In general, there are three groups of antheridiogen (Haufler and Gastony 1978; Schneller et al. 1990): antheridiogen A (produced from *Pteridium aquilinum* and reactive among members of Polypodiaceae s.l.), antheridiogen B (confined only among members of Schizaeaceae), and antheridiogen C (produced only in *Ceratopteris*). Some authors prefer to use abbreviations of the source genus to express antheridiogen names. For example, Naf et al. (1975) and Naf (1979) used AP ( = antheridiogen A), An and ALy ( = antheridiogen B, produced from *Anemia* and *Lygodium* respectively), and An (antheridiogen of *Onoclea*...
sensibilis). Other examples, such as $A_{ph}$ (produced from *Pityrogramma calomelanos*, Dubey and Roy 1985), $A_{ps}$ (secreted from *Pteris vittata*, Gemmrich 1986), and $A_{CE}$ (yield from *Ceratopteris*, Banks *et al.* 1993 and Hickok *et al.* 1995) have also been used.

The basic operative system of antheridiogen, using *Pteridium aquilinum* as a model, has been interpreted as follows (Naf *et al.* 1975; Naf 1979, and references therein). Some spores of a population germinate earlier and develop rapidly. These rapidly growing gametophytes are first to reach a developmental stage capable of producing and secreting antheridiogen which promotes antheridium formation by other slower-growing gametophytes in the neighborhood. The antheridiogen-producing gametophytes are bigger, meristem-organized and insensitive to antheridiogen by the time that they begin to produce antheridiogen, and thus differentiate only as female individuals. The smaller gametophytes stay at the male phase and never organize a meristem if antheridiogen is continuously present, but can develop into bisexual ones if not continuously exposed to effective antheridiogen concentrations. Naf *et al.* (1974) further explained that the antheridiogen is actually removing an antheridiium-block which exists intrinsically in the spore and young gametophytes, and thereafter antheridia differentiate. This antheridium-block can also be released in near-darkness without the presence of antheridiogen. Although antheridiogen can decay this intrinsic block in young gametophytes, it cannot overcome the block in older meristem-organized gametophytes which produce other inhibitors which cause themselves to be insensitive to antheridiogen. If the apical meristem is removed from the meristem-organized gametophyte, then antheridia can again be produced spontaneously (Naf 1961).

When the female gametophytes become older, they may produce proliferations which may bear antheridia on the base but usually cease producing new antheridia as a new meristem organizes at the apex of the proliferations. A simpler mechanism for antheridiogen operation
has been suggested by Korpelainen (1994) in which "antheridiogens actually affect size, and size influences sex expression".

Antheridiogen may play an important role in fern speciation and evolution, because it favors the establishment of unisexual gametophytes and thus promotes intergametophytic mating which increases the genetic diversity (Schedlbauer and Klekowski 1972; Yatskievych 1993). Schneller et al. (1990) indicated that low genetic load was related to the inability to respond to antheridiogen in Bommeria palmata. Schraudolf (1985) stated that antheridiogen activity can reach a radius of 10 cm on natural substrates and 30 cm on agar by one single gametophyte in his study of Anemia. If we consider that spore germination in the dark can be stimulated by antheridiogen, the influence of antheridiogen in outcrossing is even more obvious. However, the advantage of outcrossing caused by antheridiogen has been questioned since the relationship between the effective range of antheridiogen and the distance of sperm-travel is not well known (Willson 1981), although sperm of Athyrium filix-femina have been shown to travel about 4-8 cm (Schneller et al. 1990) and those of Equisetum may swim to 20 cm in the wild (Duckett and Duckett 1980). and to 0.3 mm/sec. for 2 hours in water under normal temperature (Bilderback et al. 1973).

The promotion of outcrossing by antheridiogen may cause an obstacle to fertilization for isolated spores. This obstacle could be solved by removing the antheridium-block through shading (near-darkness), aging and proliferation, or other possible biotic and abiotic factors. In addition, different plants express different efficiencies of response to antheridiogen among sporophyte populations (Scott and Hickok 1987) or among sporophytes in the same population (Schneller et al. 1990; Stevens and Werth 1993).

**Mating/Breeding System**

The following terminologies have been provided by Klekowski (1968; 1979) and are commonly used:
1. Intragametophytic selfing: the fusion of sperm and egg from the same gametophyte.
2. Intergametophytic selfing: the fusion of sperm and egg from different gametophytes which originated from the same parental sporophyte.
3. Intergametophytic crossing: the fusion of sperm and egg from different gametophytes which originated from different parental sporophytes.
4. Intergametophytic mating: the fusion of sperm and egg from different gametophytes which originated from unspecified sporophytes.

Intergametophytic selfing is genetically analogous to the self-fertilization of seed plants, whereas intergametophytic crossing is genetically analogous to the cross-fertilization of seed plants. Intragametophytic selfing, which cannot occur in seed plants, results in a totally homozygous zygote regardless of whether the parental sporophyte is homozygous or heterozygous. Intergametophytic selfing will produce a homozygous zygote if the parental sporophyte is homozygous, but a heterozygote is possible when the parent sporophyte is heterozygous (Klekowski 1968; Klekowski and Lloyd 1968). The events underlying homo- or heterozygous zygote production assume that no mutation occurs. However, since the origins of parental sporophytes of gametophytes are difficult to distinguish, the terminology “outcrossed mating” (outcrossing, including intergametophytic selfing and intergametophytic crossing sensu Klekowski), and “selfed mating” (or “selfing”, equal to intragametophytic selfing sensu Klekowski) have been applied (Holsinger 1990). Other similar terminologies such as “outcrossing” and “inbreeding” have also been used (e.g., Li and Haufler 1994).

Recently, based on the equivalent sexual phenotypes and breeding systems among angiosperms, gymnosperms, pteridophytes and bryophytes, Cruden and Lloyd (1995) suggested using terms such as automixis (= intragametophytic selfing), autogamy (= intergametophytic selfing), xenogamy (= intergametophytic crossing), and facultative
xenogamy (mixed breeding system) so as to improve communication among plant systematists.

Klekowski (1968) also distinguished the mating system and breeding system. The former uses predictive parameters, such as gametangium sequence, gametangium arrangement, incompatibility, etc., to estimate the probability of intra- or intergametophytic mating. The latter uses demonstrative characteristics, such as genetic load or lethal genes, to estimate the level of heterozygosity and thus indicate if it is an inbreeder or outbreeder. Studies of breeding systems usually use isolated-spore cultures, but recently electrophoresis has become a more powerful tool (Haufler 1987).

Gametangium sequence
Klekowski (1968; 1969a) classified the sexual expression sequence into four types:
(A) Male -> Hermaphrodite (an adaptation for intragametophytic selfing);
(B) Male -> Female -> Hermaphrodite (favorable for intergametophytic mating);
(C) Female -> Hermaphrodite (higher possibility of intergametophytic mating);
(D) Male -> Hermaphrodite -> Female -> Hermaphrodite (both intra and intergametophytic mating depending on the length of the phase).

He also listed some examples corresponding to the above types.

Masuyama (1975a, b) reported that some species did not fit into Klekowski's catalogue due to the effect of environmental factors, and reclassified sexual expression into three types:
(A) Male -> Female or only female (adaptation to intergametophytic mating, the latter exists only in very favorable growth conditions).
(B) Male -> Hermaphrodite, or Female -> Hermaphrodite (the later occurs under favorable growth conditions, this system favors intragametophytic selfing but intergametophytic mating is possible).
(C) Male -> Hermaphrodite -> Female (promote intragametophytic selfing initially and intergametophytic mating later).

Because most polyploids have type B, Masuyama (1975b) concluded that type B is derived from type A in the evolution process. Lloyd (1974a) argued that the correlation of the gametangium sequence and the mating system might be too simplistic, since other factors, such as lethal genes, antheridiogens, and environmental factors, affect the reproductive behavior.

**Self-incompatibility**  
Self-incompatibility has been documented only in *Pteridium aquilinum* (Wilkie 1956), but Klekowski (1972b) used the model proposed by Mather (1942) to argue that the high self-sterility was due more to genetic load and concluded that the self-incompatibility in *P. aquilinum* was not true for the entire species. However, Lovis (1977) indicated that Wilkie's interpretation of his data was likely right, since Wilkie's materials were tetraploid and since "incompatibility systems originally established and efficient at the diploid level are less effective in polyploid derivatives", and thus "a weak incompatibility system" might exist in Wilkie's materials.

**Homoeologous Chromosome pairing**  
Homosporous fern gametophytes are potentially bisexual and thus intragametophytic mating seems likely. However, inbreeding has been thought to be an evolutionary dead end since the resulting homozygosity leads to low genetic diversity. Klekowski and Baker (1966) reported that relatively high chromosome numbers and high frequency of polyploids were prevalent in homosporous ferns. They thus proposed that the duplication of loci should help maintain heterozygosity despite the effects of self-fertilization. Later, Klekowski (1972a; 1973b) proposed a hypothesis that meiosis in allopolyploid ferns allows homoeologous chromosome pairing and produces genetically heterogeneous spores. Therefore, polyploidy functions to store the genetic heterozygosity resulting from intragametophytic selfing, and the homoeologous pairing serves to release the
genetic variability during intragametophytic selfing. At present, evidence of homoeologous
pairing has most clearly been documented in *Ceratopteris* (Klekowski and Hickok 1974;
Hickok 1978), and claimed for a few other species (Klekowski 1976; Bierhorst 1975;
Chapman *et al.* 1979). However, homoeologous pairing as a regular phenomenon is doubted
by most researchers.

For example, Lovis (1977) criticized the cytological evidence of homoeologous
pairing provided by Klekowski and Hickok (1974) and Bierhorst (1975) by pointing out that
other chromosome behavior could result in the same pairing patterns. Furthermore, the
multiple electrophoresis bands expressed in studies by Chapman *et al.* (1979) have been
interpreted as the outcome of phenomena other than homoeologous pairing. For example,
Gastony and Gottlieb (1982; 1985) demonstrated that multiple bands were produced from
outcrossed heterozygosity coded by alleles at a single locus and questioned the necessity of
homoeologous pairing to produce such patterns. Gastony and Darrow (1983) found that the
duplicated bands of diploid *Athyrium felix-femina* were products of subcellularly localized
isozymes from the cytoplasm and chloroplasts, and questioned again the evidence of
Chapman *et al.* (1979). Haufler and Soltis (1983) and Haufler (1985) further argued that the
species *Pteridium aquilinum*, studied by Chapman *et al.* (1979), might be a paleopolyploid
which has been diploidized and expressed as a functional diploid which did not show
homoeologous sets. Subsequently, Wolf *et al.* (1987) used isozyme electrophoresis data to
demonstrate a genetically diploid level in their material of *P. aquilinum*, and again doubted
the conclusion of Chapman *et al.* (1979).

Based on the relatively scarce evidence of homozygous sporophytes in most natural
ferns, Ganders (1972) suggested that although polyploidy may function to counteract
complete homozygosity and to retain genetic diversity, natural selection against
homozygosity is a more probable mechanism for maintaining heterozygosity. Therefore, the
homoeologous pairing in homosporous ferns has been considered to be a possibly occasional event rather than a regular characteristic (C. J. Peck 1985).

*Genetic load* The degree of intragametophytic selfing can be calculated as genetic load (or presence of lethal or deleterious genes) which may be expressed in different stages of gametophytes and sporophytes (called gametophytic load and sporophytic load respectively by Klekowski 1984). Traditionally, genetic load has been measured by isolated-spore (or gametophyte) cultures, in which only intragametophytic selfing (or apogamy which can be detected by careful observation) is possible, and only for hermaphroditic gametophytes. The percentage of bisexual individuals not producing sporophytes in the isolated culture is an estimate of the genetic load (Lloyd 1974a; Klekowski 1979). Accordingly, the higher the value of the genetic load, the lower the possibility of successful intragametophytic selfing. Klekowski (1968 and in all of his following studies) did not water a set of control plants in order to measure the number of sporophytes produced by apogamy.

Two sources of genetic load are relevant in ferns: mutational and heterotic load (Klekowski 1984). The former results from new mutations whereas the later is maintained in the population in heterozygous individuals (Klekowski 1984). Although Lovis (1977) believed that heterotic load is the majority of the genetic load in ferns, Klekowski (1984) concluded that mutational load was also present in gametophytes of *Onoclea sensibilis* and *Matteuccia struthiopteris*. Klekowski (1985) thus stated that mutational load is possibly more important in the majority of ferns. Hedrick (1987a) suggested that heterotic load was low in inbreeding systems. Nevertheless, except for a few studies, most research measures genetic load without considering whether it is predominantly mutational or heterotic load.

Genetic load studies have involved more than 60 species and these have been listed in some review papers (C. J. Peck 1985; Raghavan 1989). In these studies, the presence or absence of sporophyte lethals has been determined by sporophyte formation, thus only a
portion of the genetic load, *i.e.*, gametogenesis lethal, fertilization lethal, and early embryo development lethal, is detected (Ganders 1972; Klekowski 1982). Lethals or deleterious alleles expressed in older stages of sporophyte development have also been shown to exist (Schneller 1979; Peck *et al.* 1990).

Depending on the species, genetic load varies from 0%, *e.g.*, *Doodia aspera*, *D. caudata*, and *Woodwardia fimbriata* (Klekowski 1969b), *Pityrogramma calonelanos* (Singh and Roy 1977), *etc.*, to 100%, *e.g.*, *Woodwardia japonica* (Klekowski 1969b), *Bommeria hispida*, *B. subpaleacea* (Haufler and Gastony 1978), *Dryopteris ludoviciana* (Cousens 1969), and so on. Genetic load may also vary among different populations within a species, *e.g.*, *Pteridium aquilinum* (Wilkie 1956; Klekowski 1972b), *Blechnum spicant* (Cousens 1979), *etc.*, or among different sporophytes of the same population, *e.g.*, *Cibotium glaucum* (Lloyd 1974a, b).

Genetic load may be related to the habitats of species. Lloyd (1974b) found that the genetic load of species growing in intermediate and mature rainforest was higher, whereas that of pioneer species was lower and thus favored the establishment of a new population through an isolated spore dispersal in lava habitats in Hawaii.

Genetic load may also correlate with population size. Klekowski (1970a) found that there was no genetic load in a small population in Hawaii of the species *Ceratopteris thalictroides*. On the other hand, 55.5% of genetic load was found in a larger population of the same species in Hawaii (Klekowski's observation, cited from Lloyd 1974a). In *Osmunda regalis*, Klekowski (1976) reported that the highest genetic load existed in the largest population tested.

In addition, genetic load is related to ploidy, *i.e.*, diploid species tend to have higher genetic loads and favor intergametophytic mating whereas polyploid species tend to have
lower genetic loads and favor intragametophytic selfing (Masuyama 1979; Masuyama et al. 1987; Masuyama and Watano 1990; Hedrick 1987b).

**Isolate potential** C. J. Peck (1985) and Peck et al. (1990) defined several reproductive terminologies: germination-development potential, bisexual potential, selfing potential, genetic load, and gametophyte isolated potential, and used spore isolate potential to summarize all of the above spore-gametophyte reproductive potentials. Accordingly, higher isolate potential is probably related to the higher rate of intragametophytic selfing and thus represents higher potential for successful long-distance dispersal. These parameters have also been documented for 14 species of Woodman Hollow Preserve in Iowa.

**Electrophoresis** Starch gel enzyme electrophoresis has been demonstrated to be an effective method to study the genetics of polyploidy and to examine the breeding systems of diploid ferns (Haufler 1987; D. E. Soltis and P. S. Soltis 1987a; P. S. Soltis and D. E. Soltis 1989, 1990). The homosporous Pteridophyta have been recognized as possessing unusually high chromosome numbers. The average chromosome number of n = 57.05 is in contrast to that of angiosperms and heterosporous Pteridophyta (n = 15.99 and 13.62 respectively) (Klekowski and Baker 1966). Thus homosporous ferns have also been assumed to be highly polyploid (Klekowski 1973b, 1979; Love et al. 1977; Wagner and Wagner 1980). However, electrophoretic evidence has indicated that many ferns are functionally (or genetically) diploid despite their high chromosome numbers (Gastony and Darrow 1983; Werth et al. 1985; Hauffer and Soltis 1986; Li and Hauffer 1994). Possible explanations are that species have originated by allopolyploidy followed by chromosome diploidization through gene silencing, ancient autopolyploidy, or initial high chromosome numbers (Hauffer and Soltis 1986; Hauffer 1987; D. E. Soltis and P. S. Soltis 1987a). Furthermore, Vida (1976) estimated that there is only about 43.5% polyploidy in ferns, compared to 47% polyploidy in angiosperms (Grant 1981). Klekowski (1973b) suggested that polyploidy has been selected in
homosporous ferns as a mechanism to maintain variability. Others have considered the significance of this role to be overestimated (Haufler 1987). To maintain genetic variation, homosporous ferns appear to behave in the same way as angiosperms, mainly through various levels of outcrossing, instead of homoeologous pairing (D. E. Soltis and P. S. Soltis 1987a).

Due to the potential for bisexual gametophytes and the genetic buffering provided by high chromosome number, self-fertilization has been considered to be adaptive and widespread among homosporous ferns (Klekowski and Baker 1966; Klekowski 1973b). Although some isozyme electrophoretic data have supported this point (Werth et al. 1985; McCauley et al. 1985; D. E. Soltis and P. S. Soltis 1986; Watano and Sahashi 1992), other electrophoretic evidence has shown high level of heterozygosity in natural populations indicating outcrossing (Haufler and Soltis 1984; Gastony and Gottlieb 1985; P. S. Soltis and D. E. Soltis 1987) and mixed mating (D. E. Soltis and P. S. Soltis 1987 b; P. S. Soltis and D. E. Soltis 1988; Ranker 1992b) to be the prevalent breeding behavior. D. E. Soltis and P. S. Soltis (1992) reviewed 20 previously studied species and found that most homosporous ferns were extreme outcrossers. Nearly all exclusive inbreeders are found in a few species that possess subterranean gametophytes, e.g., Botrychium (McCauley et al. 1985; D. E. Soltis and P. S. Soltis 1986), Ophioglossum (McMaster 1994), Sceptridium (Watno and Sahashi 1992), although outcrossing may sometimes happen in the underground gametophytes due to crowded gametophytes resulting from "spore showers" (Wagner et al. 1985). Haufler (1987) concluded that inbreeding is possibly a derived mechanism for facilitating sporophyte establishment by isolated spores.

There are two common ways to estimate the breeding system of ferns with the use of isozyme electrophoresis (P. S. Soltis and D. E. Soltis 1990). The first is the "intragametophytic selfing rate" using genotypic frequencies in sporophytes (Holsinger
1987), in which the value 1 indicates that all sporophytes are produced through intragametophytic selfing, and the value 0 indicates that no selfing occurs at all. The second parameter is the "fixation index", or inbreeding coefficient (Wright 1965). A high negative value (to -1) indicates high levels of heterozygosity, suggesting obligate outcrossing, whereas a high positive value (to +1) indicates higher level of homozygosity and predominant inbreeding. A value of 0 is expected in random breeding. However, in natural populations, the rate of outcrossing may be higher than estimated by the level of heterozygosity since, in addition to self-fertilization, homozygous sporophytes may be produced through outcrossing between identically homozygous gametophytes (Gastony and Gottlieb 1985; McMaster 1994), or result from founder effect in isolated population and/or limited gene flow among populations (Li and Haufler 1994).

**Literature cited**


1. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF *CAMPYLOGNEURIUM ANGUSTIFOLIUM* (SWARTZ) FEE

This paper is prepared for publication in American Fern Journal

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**Introduction**

The morphology of fern gametophytes has been studied and applied to taxonomy and systematics for a long time. Beyond the standard heart shapes depicted in most textbooks, great variations have also been shown (Atkinson 1973; Nayar and Kaur 1971). These variations include the ability to grow indeterminately and branch so that perennial gametophyte clones of considerable size may be produced (Farrar 1990; Dassler 1995). This morphological diversity may relate to the species' mating system and reproductive biology.

Four types of mating system in homosporous pteridophytes have been defined by Klekowski (1979): (1) intragametophytic selfing, (2) intergametophytic selfing, (3) intergametophytic crossing, and (4) intergametophytic mating. Among these mating systems, intragametophytic selfing has been suggested previously as being predominant in homosporous ferns due to the close proximity of antheridia and archegonia on bisexual gametophytes (Klekowski and Baker 1966; Klekowski 1973, 1979) and indeed there are some homosporous ferns which have very high rates of intragametophytic selfing (Crist and Farrar 1983; McCauley *et al.* 1985; Soltis and Soltis 1986a; Watano and Sahashi 1992). Polyploid species may also favor intragametophytic selfing due to duplicated alleles which mitigate the problem of recessive deleterious allele expression associated with selfing (Klekowski and Baker 1966; Lloyd 1974a; Masuyama and Watano 1990). Recently, however, electrophoretic evidence has shown that intergametophytic mating is the most common breeding system in homosporous ferns (Haufler and Soltis 1984; Holsinger 1987; Soltis and Soltis 1986b; Soltis and Soltis 1990; Ranker 1992).
Several types of reproductive behavior can reduce the probability of intragametophytic selfing. Asynchronous maturation of male and female gametangia is one way to avoid intragametophytic selfing in bisexual gametophytes (Klekowski 1968, 1969; Masuyama 1974a, b). Possession of an antheridiogen system is another way to facilitate production of unisexual gametophytes, and thus promote intergametophytic mating (Naf et al. 1975; Voeller 1971; Hamilton and Lloyd 1991). Antheridiogen (or germinin, proposed by Voeller 1971) also overcomes the inhibition of spore germination in darkness (Voeller 1971; Schneller et al. 1990). However, except for Polypodium australe (Welling and Haufler 1993), and Aglaomorpha meyeniana (Naf 1966; 1969), Polypodiaceae s.s. has been thought neither to produce its own antheridiogen nor to respond to the antheridiogen of Pteridium aquilinum (L.) Kuhn, the most common antheridiogen known (Voeller 1971; Raghavan 1989). Genetic load is also an obstacle to successful intragametophytic selfing, and the degree of genetic load has been used to measure the sporophyte heterozygosity and probable breeding system (Lloyd 1974a; Cousens 1988). However, most of the information known about the reproductive biology of ferns is from research on terrestrial ferns which encounter very different environmental events from epiphytic ferns.

Campyloneurum angustifolium (Swartz) Fee, a member of Polypodiaceae, distributed from Florida and Mexico to Central and South America (Nauman 1993), is a rare epiphytic species in Florida (Lellinger 1985). The gametophyte morphology has been previously described (Nayer 1962) but the reproductive biology of this species has not been mentioned. Due to the epiphytic habitat and the nature of its bark and bryophyte substrate, dispersed spores of this species are likely to be more widely separated, and the interaction of the resulting gametophytes more hindered than in terrestrial species (Dassler 1995). Thus gametophytes of epiphytic species might be more likely to produce their sporophytes through intragametophytic selfing. This paper will investigate this issue in C. angustifolium,
addressing the following questions: (1) how are gametangia expressed in this species, (2) how much genetic load exists, (3) do gametophytes produce or/and respond to antheridiogen, (4) through which mating system are sporophytes produced, (5) how does gametophyte morphology relate to the reproductive system.

Materials and methods

Two sources of Campyloneurum angustifolium were used. One source was plants of unknown origin in the greenhouse of the Department of Botany, Iowa State University (represented by "A"). The other source (represented by "B") was plants collected in August, 1994, from Marie Selby Botanical Garden, Sarasota, Florida (Chiou 14337). Because antheridiogen of Pteridium aquilinum (Apt) has been well studied (Naf et al. 1975), and gametophytes of Onoclea sensibilis L. have been used to "safely identify antheridium-inducing activity" due to their complete lack of spontaneous antheridium production in young stages (Naf 1956), these two species were also used in this study to assay the activity of antheridiogen of Campyloneurum angustifolium. Spores of Onoclea sensibilis were collected from Marshall County, Iowa, in September, 1994 (Chiou s.n.), and those of Pteridium aquilinum were in part collected in June, 1991 from Iowa City, Iowa (Farrar 91-6-26-27), and in part kindly sent by Dr. David Wagner, collected from Eugene, Oregon, in August 3, 1994. Voucher specimens of C. angustifolium were deposited in the Ada Hayden Herbarium (ISC) of the Department of Botany at Iowa State University. Spores obtained from fertile fronds (Chiou and Farrar 1994) were stored in the refrigerator at about 5°C.

Spores were sown on 1% agar-solidified media which contained Bold's macronutrients (Bold 1957) and Nitsch's micronutrients (Nitsch 1951), and a trace of ferric chloride (Peck 1985). Cultures were maintained under continuous, white fluorescent illumination of 2000-3000 lux. Temperature was maintained between 20-24°C.
Gametophyte morphology was observed under dissecting and compound microscopes. Pictures were either taken by auto-camera or hand-drawn using a drawing tube. Sizes of gametophytes were measured as the width of the widest part. Gametophytes were observed every half month.

To harvest antheridiogen-enriched solution, the following procedure was used. One mg of spores of *C. angustifolium* and *P. aquilinum* was separately sterilized with 10% Clorox for 5 minutes and then rinsed with sterilized water. The sterilized spores were then suspended in 2 ml of sterilized water. Two drops of the spore suspension were placed into each of several petri dishes of 8.5 cm diameter containing about 50 ml of agar media. The cultures of *C. angustifolium* and *P. aquilinum* were frozen at 8 months and 2 months old respectively, then thawed and the liquid extract collected. 2% and 10% dilutions of the extract were made by adding 20 drops (ca. 1 ml) of the extract into large petri dishes containing 50 ml of agar medium and to small petri dishes containing 10 ml of agar medium respectively. In addition to these extracts, GA3 (5x10^-5 g/ml) was also used to test antheridiogen response.

Considering the possibility that some antheridiogens might be short-lived (Emigh and Farrar 1977), that the time of harvesting extract might be not proper, or that the harvesting method may destroy antheridiogen activity, three additional experiments were conducted in which agar with mature growing gametophytes was used directly for the assay. In the first experiment, the agar with growing mature gametophytes (8-month- and 2-month-old of *C. angustifolium* and *P. aquilinum* respectively) was cut into 1 x 2 cm blocks. Then each agar block was turned on its side (turned block), so the gametophytes stood on one side of the agar block. Ten spores were sown on the top of each block, and ten such blocks were put into each of two petri dishes. In the second experiment, agar supporting mature gametophytes (*C. angustifolium* gametophytes were 1 year old) was cut into four parts. Each part was placed upside down in another petri dish, and spores were sown on the upside-down agar (inverted
block). In the third experiment, some of the gametophytes were removed from cultures (*C. angustifolium* gametophytes were 1 year old) to make a 1 cm width lane (cleared block), and spores were sown on these cleaned areas (between mature gametophytes). A complete randomized design was used. Preparations of both extracts and blocks with growing gametophytes were used in tests of germination capacity in darkness and promotion of antheridium formation in the light. The percentage of plants forming antheridia was counted as the number of male plus bisexual individuals. Tests were repeated twice in the first experiment (Tables 5, 7) and four times in the second and third experiments (Tables 6, 8). At each observation time, 25 gametophytes were removed from each dish in the first experiment and 12 gametophytes were removed from each dish in the second and third experiments.

Capability of spore germination in darkness and the promotion of spore germination in the dark by antheridiogen were tested by wrapping two layers of aluminum foil around spore-sown petri dishes. These wrapped dishes were placed in a dark cabinet and unwrapped and examined one month later. Tests were repeated two (table 4) and three (table 5) times. Germination rates were determined by counting 100 spores; the proportion of male plants was measured as the number of male gametophytes divided by the total number of dark-germinated gametophytes. A complete randomized design was used.

Genetic load was calculated by comparing isolated-spore and isolated-gametophyte cultures with paired-spore and paired-gametophyte cultures. These cultures were grown on "jelly-mold" plates, each with 20 cells. Each cell contained about 6 ml of agar medium. In each plate, a single spore was transferred onto each of 5 cells, and a one month old gametophyte which was still asexual was transferred onto each of another 5 cells. Another 10 cells were used as paired-spore and paired-gametophyte controls. Five such plates were separated by transparent plastic sheets and stacked into transparent plastic vegetable crispers for a total of 50 replicates of each culture type. The light intensity of these cultures was
maintained between 1500 lux (the bottom layer) and 3500 lux (the top layer). Plants were watered every two weeks after gametophytes were 4 months old. Genetic load was measured by counting the percentage of bisexual gametophytes failing to produce sporophytes. The five layers of plates were designated as blocks, and spore-culture vs. gametophyte-culture were designated as a split plot. Sporophytes were determined to have been produced sexually by examination with a compound microscope. Since two source of spores were used, a Latin Square was designed. Five treatments were "A", "B" (isolated-cultures of two different sources), "AA", "BB" (paired-cultures of two same sources), and "AB" (paired-cultures of different sources). Sporophytes were determined to have been produced sexually by examination with a compound microscope.

Results

Morphology

In multispore cultures, the rhizoid was usually the first cell produced on germination (Fig. 1) about one week after sowing spores. The basal cell underwent a second division (Fig. 2), resulting in a protonemal cell which subsequently produced a filament by serial divisions (Fig. 3). The lengths of filaments were various, usually two to six cells. In addition to the basal cell, rhizoids also emerged from other cells of the filament below the top cell. A longitudinal division usually occurred at the apical cell and/or the other cells of the filament (Figs. 4, 5). Sometimes all the filament cells except the basal cell eventually divided longitudinally (Fig. 7). Subsequent divisions and expansion of filament cells formed a broad spathulate prothallus (Fig. 6). An obconical meristematic cell was built at the anterior margin of the thallus by two oblique divisions (Fig. 7). A pluricellular meristem replaced the single meristematic cell later, and a symmetrical (or nearly) cordate gametophyte eventually formed (Fig. 8).
Figs. 1-16. Gametophyte development in *Campyloneurum angustifolium*. Figs. 1-8. Germination and early development. Fig. 9. Male gametophyte. Fig. 10. Hermaphroditic gametophyte. Fig. 11. Branched female gametophyte. Fig. 12. Part of branched hermaphroditic gametophyte. Figs. 13-16. Hairs. An = antheridium. Ar = archegonium.
In addition to growing on the margin, rhizoids also occurred on both dorsal and ventral sides of the midrib as well as the wings, but those on the latter were fewer than those on the midrib or on the margin.

After attaining late spathulate or early cordate stage, gametophytes produced hairs, most of which were unicellular, papillate, and secretory. These first appeared on the margins (Figs. 8-12), then on both dorsal and ventral sides of wings and midribs (Figs. 10-12). On relatively old gametophytes, some hairs became bigger (Fig. 13) and some multicellular hairs appeared (Figs. 14-16). A secretion always capped the glandular hairs.

At about 2.5 months, antheridia appeared, usually on the posterior part (Figs. 9, 10, 12), or sometimes near the apical meristem if a midrib had not formed, or on the margin (Fig. 10). Archegonia, appearing at about 1.5 months, were distributed on the midrib behind the meristem (Fig. 10-12).

As gametophytes grew older, their wings became lobed and sections of the margin became active and produced additional meristems (Figs. 11, 12). From these meristems, branches formed and produced additional meristems. Branches arising from one or a few cells also occurred. A mat of overlapping branches thus formed (Fig. 28). Gametophytes continued growing over two years and several sporophytes were derived from many of the individual gametophytes which still remained growing after sporophyte production.

**Sex sequence**

In multicultures, gametangia did not appear until 1.5 months after sowing spores. Sexual frequency varied with the culture time (Table 1). Archegonia developed earliest and female plants remained most common during the culture period of 3 months. Males and hermaphroditic plants appeared at 2.5 months. Males were relatively small (Table 1) and seemed to be developing from slow growing individuals, perhaps late-germinated spores.
Figs. 17-27. Gametophyte response to experimental treatments. Figs. 17-24. *Campyloneurum angustifolium*. Fig. 17. Dark-growing gametophyte with 2 antheridia derived from a spore germinated under darkness on an inverted block of *Pteridium aquilinum* gametophytes. Fig. 18. 2 month old male gametophyte growing on and inverted block of *P. aquilinum* gametophytes. Fig. 19. 2 month old male gametophyte growing on a turned block of *P. aquilinum* gametophytes. Fig. 20. 2.5 month old male gametophyte growing on an inverted block of *C. angustifolium* gametophytes. Fig. 21. 2.5 month old male gametophyte growing on a cleared block of *C. angustifolium* gametophytes. Fig. 22. 2.5 month old male gametophyte growing on a cleared block of *P. aquilinum* gametophytes. Fig. 23. 3 month old male gametophyte growing on an inverted block of *C. angustifolium* gametophytes. Fig. 24. 3 month old gametophyte growing on a cleared block of *C. angustifolium* gametophytes. Figs. 25-27. *Onoclea sensibilis*. Fig. 25. 0.5 month old gametophyte of *Onoclea sensibilis* growing on an inverted block of *C. angustifolium* gametophytes. Fig. 26. 1.5-month-old gametophyte of *O. sensibilis* growing on 10% extract of mature *C. angustifolium* gametophyte cultures. Fig. 27. 1.5 month old gametophyte of *O. sensibilis* growing on a cleared block of *C. angustifolium*. An = antheridium.
Figs. 28-32. Gametophytes of *Campyloneurum angustifolium*. Fig. 28. One 6-month-old clonal gametophyte. Fig. 29. From a dark-germinated spore sown on a cleared block of *C. angustifolium*. Fig. 30. From a dark-growing gametophyte with 2 gametangia on an inverted block of *P. aquilinum* gametophytes. Fig. 31. A 2 month old male gametophyte growing on an inverted block of *P. aquilinum* gametophytes. Fig. 32. A 2 month old male gametophyte with released sperms growing on a cleared block of *P. aquilinum* gametophytes. Bar = 1 mm for Fig. 28; bar = 0.1 mm for Figs. 29-32.
Hermaphrodites were relatively large (Table 1) and seemed more likely derived from female plants.

The percentages of males and hermaphrodites were not significantly different either between the isospore- and isogametotophyte cultures or between the two spore sources, at 8-month after sowing spores (Table 2). On the other hand, among isolated-cultures, the percentages of the male and hermaphrodite of gametophytes on the plates of the bottom layer were significantly different from those on other layers (Table 2). The proportion of males was more than half of the population on the bottom layer, whereas most of the gametophytes growing on the plates of the other layers were hermaphrodites with no significant difference among them (Table 2).

**Genetic load**

As shown in Table 3, gametophytes of the source "B" totally failed to produce sporophytes in both the isolated-spore and isolated-gametophyte cultures at 8-month after spore sowing. Thus the genetic load was 100, and there was not any difference between the spore-culture and gametophyte-culture treatments. On the other hand, gametophytes of the

<table>
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<td></td>
<td>A  M  F  H*</td>
<td>A  M  F  H</td>
<td>A  M  F  H</td>
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<tr>
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<td>29  -  71  -</td>
<td>27  4  63  6</td>
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* A = asexual, M = male, F = female, H = hermaphrodite.
Table 2. The sex expression (%) of *Campyloneurum angustifolium* in isolated-spore (spore) and isolated-gametophyte (gametophyte) cultures at 8 months after sowing spores.

<table>
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<th>Female</th>
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<td>81(a)</td>
</tr>
<tr>
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<tr>
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<td>92(a)</td>
</tr>
<tr>
<td>B</td>
<td>16(a)</td>
<td>0</td>
<td>84(a)</td>
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<tr>
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</tr>
<tr>
<td>1</td>
<td>6(b)</td>
<td>0</td>
<td>94(a)</td>
</tr>
<tr>
<td>2</td>
<td>0(b)</td>
<td>0</td>
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</tr>
<tr>
<td>3</td>
<td>6(b)</td>
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<td>94(a)</td>
</tr>
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</tbody>
</table>

1,2,3,4,5,6 The same letter in parentheses in each block means no significant difference in Duncan's multiple test (95% c.l.).

7 Layer 1 is located on the top, layer 5 is on the bottom.

Table 3. The percentage of sporophyte production and the genetic load (by surviving bisexual gametophytes) expressed by 8 month old *C. angustifolium* in spore-cultures (spore) and gametophyte-cultures (gametophyte) from two sources.

<table>
<thead>
<tr>
<th>Source</th>
<th>% sporophyte</th>
<th>load</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spore2</td>
<td>gametophyte2</td>
</tr>
<tr>
<td>A</td>
<td>77(a)</td>
<td>90(a)</td>
</tr>
<tr>
<td>AA</td>
<td>92(a)</td>
<td>96(a)</td>
</tr>
<tr>
<td>AB</td>
<td>73(ab)</td>
<td>86(a)</td>
</tr>
<tr>
<td>BB</td>
<td>44(c)</td>
<td>48(bc)</td>
</tr>
<tr>
<td>B</td>
<td>0(d)</td>
<td>0(d)</td>
</tr>
</tbody>
</table>

1 "A", "B", isolated culture; "AA", "AB", "BB", paired culture; "A" from greenhouse of Department of Botany, Iowa State University; "B" from Marie Selby Botanical Garden, Sarasota, Florida.

2-5 The same letter in parentheses at each of the items indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 9.28, 3.88, and 5.50, and 2.46 for 2, 3, 4, and 5 respectively.
isolated-cultures from source "A" and all the paired-cultures were relatively successful in producing sporophytes. Compared to that of "B", the genetic load of "A" was low (12 on average). On average, the gametophytes from "A" produced sporophytes more successfully than those from "B", no matter whether they were in isolated or paired cultures.

**Spore germination in darkness**

Spores of *Campyloneurum angustifolium* did not germinate in the dark or in treatments of where 2% culture extract of *Pteridium aquilinum* or *C. angustifolium* gametophytes was added, on turned blocks (Table 4), or on media with GA3, 10% extractions of *C. angustifolium* gametophyte cultures (Table 5). Spores of *C. angustifolium* did germinate in the dark when 10% extract of *P. aquilinum* gametophyte cultures was added to the medium, and when spores were sown on inverted culture media of mature gametophytes of *C. angustifolium* or *P. aquilinum* or beside them (Table 5). The germination percentages in darkness were indeed very low, but some spores did germinate in those treatments whereas none germinated in the dark controls. In general, the germination rates of *C. angustifolium* in the treatments of *P. aquilinum* gametophytes were higher than those of *C. angustifolium* gametophytes (Table 5). Among these dark-germinated gametophytes, only those growing near mature *P. aquilinum* gametophytes had produced antheridia when they were observed (Table 5, conditions 5, 6; Figs. 17, 30), and the vegetative prothallus cell was often relatively elongated (Fig. 30). Some dark-germinated gametophytes were sterile (Fig. 29), perhaps still too young when observed.

**Antheridium promotion in *Onoclea sensibilis***

Gametophytes of *Onoclea sensibilis* were significantly promoted to form antheridia at 0.5 months if the spores were sown near mature gametophytes of *C. angustifolium*, whereas all of them remained asexual under the control condition (Tables 6, 7). At one month and 1.5
Table 4. Average dark germination percentage and male percentage of *C. angustifolium* at one month after sowing spores under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Germination %</th>
<th>Male %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Light control</td>
<td>93</td>
<td>0</td>
</tr>
<tr>
<td>2. Dark control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3. Ca 2% extract</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4. Ca turned block</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5. Pt 2% extract</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6. Pt turned block</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Ca = *C. angustifolium*, Pt = *P. aquilinum*.

Table 5. Average dark germination percentage and male percentage of *C. angustifolium* at one month after sowing spores under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Germination %</th>
<th>Male %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Light control</td>
<td>77.0**</td>
<td>0**</td>
</tr>
<tr>
<td>2. Dark control</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>3. Ca inverted block</td>
<td>0.7</td>
<td>0</td>
</tr>
<tr>
<td>4. Ca cleared block</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>5. Pt inverted block</td>
<td>1.3</td>
<td>72</td>
</tr>
<tr>
<td>6. Pt cleared block</td>
<td>2.0</td>
<td>67</td>
</tr>
<tr>
<td>7. Ca 10% extract</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>8. Pt 10% extract</td>
<td>1.3</td>
<td>0</td>
</tr>
<tr>
<td>9. GA3 5x10^-5 g/ml</td>
<td>0.0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Ca = *C. angustifolium*, Pt = *P. aquilinum*.

** SEM = 0.55, 22.30 for germination % and male % respectively, for conditions 3-8.

months after spores were sown, 2% and 10% extracts of *C. angustifolium* gametophyte cultures also significantly enhanced the antheridium production. A few of the gametophytes growing under the control condition produced antheridia by that time but significantly less than those growing under treated conditions (Tables 6, 7). The influence of the extract (either 2% or 10%) was slightly less than or not significantly different from those on agar containing secretion from living gametophytes (Table 6, condition 2 vs. condition 3; Table 7, condition 2 vs. conditions 3, 4). Antheridia were produced from very young filaments (Fig. 25) or on
Table 6. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Period</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 months</td>
<td>1 month</td>
<td>1.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (b)**</td>
<td>2 (b)</td>
<td>10 (c)</td>
</tr>
<tr>
<td>2. Ca. 2% extract</td>
<td>2 (b)</td>
<td>58 (a)</td>
<td>68 (b)</td>
</tr>
<tr>
<td>3. Ca. turned block</td>
<td>10 (a)</td>
<td>80 (a)</td>
<td>100 (a)</td>
</tr>
</tbody>
</table>

* Ca = *C. angustifolium*.

** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 1.41, 7.62, and 8.49 for 0.5 months, 1 month, and 1.5 months respectively, for conditions 2 & 3.

Table 7. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Period</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 months</td>
<td>1 month</td>
<td>1.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (b)**</td>
<td>6 (c)</td>
<td>17 (c)</td>
</tr>
<tr>
<td>2. Ca 10% extract</td>
<td>0 (b)</td>
<td>69 (b)</td>
<td>79 (b)</td>
</tr>
<tr>
<td>3. Ca cleared block</td>
<td>86 (a)</td>
<td>96 (a)</td>
<td>96 (a)</td>
</tr>
<tr>
<td>4. Ca inverted block</td>
<td>75 (a)</td>
<td>86 (ab)</td>
<td>94 (a)</td>
</tr>
</tbody>
</table>

* Ca = *C. angustifolium*.

** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 9.77, 6.90, and 4.56 for 0.5 months, 1 month, 1.5 and months respectively, for conditions 2-4.

spathulate through heart-shaped stages of prothallus development (Figs. 26, 27). Usually only one or two antheridia were produced at the young stages, but they were more abundant on the relatively mature gametophytes (Figs. 25-27) and active sperms were released.

**Antheridium promotion in *Campyloneurum angustifolium***

GA3, all culture extracts of *C. angustifolium* or *P. aquilinum* gametophytes, or the secretions of 8-month-old living gametophytes of *C. angustifolium* did not promote
antheridium formation in *C. angustifolium* during the 3-month culture period (Tables 8, 9). On the other hand, the secretion of living gametophytes of one year old *C. angustifolium* significantly promoted antheridium formation 2.5 months after sowing spores, and the secretions of living gametophytes of *P. aquilinum* promoted antheridium onset significantly 2 months after sowing spores. The secretions of one year old living gametophytes of *C. angustifolium* promoted antheridium development less than did those of *P. aquilinum* in both of the number of male gametophytes (Tables 8, 9) and number of antheridia on the gametophyte (Figs. 22, 32 vs. Figs. 20-21, 23-24). In addition, the secretions of the former usually promoted antheridium formation on the late spatulate or early cordate gametophytes (Figs. 20, 21, 23, 24), whereas the secretions of the latter usually induced the antheridium formation on very young and relatively small gametophytes (Figs. 18, 19, 22, 31, 32). Gametophytes induced to produce antheridia precociously released abundant active sperms at 2 and 2.5 months (Fig. 32), and always remained relatively small. Sperms released from 3-month-old gametophytes swam relatively sluggishly, and these gametophytes gradually became brown and died while the control gametophytes still grew well.

**Discussion**

The gametophyte of *Campyloneurum angustifolium* has been previously described as cordate and as Drynaria-type in development (Nayar 1962; Nayar and Kaur 1971). In this observation, the Drynaria-type of development was also found, but the cordate gametophytes subsequently became branched to form a clonal mat of gametophytes. Possibly Nayar’s (1962) observation was not long enough to observe branching. Gametophyte clones, formed by repeated branching and vegetative propagation, may function to increase the physical living space occupied and to prolong their life span. These extended clonal and perennial gametophytes continuously form gametangia on their new proliferations and thus enhance the possibility of interaction with other gametophytes established previously or later.

Intergametophytic mating recently has been thought to be the most common breeding system in homosporous ferns, and genetic load has been suggested to be the primary mechanism promoting outcrossing (Masuyama 1979, 1986; Haufler *et al.* 1990; Haufler and
Table 8. Average antheridium formation (%) by *Campyloneurum angustifolium* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
<th>2 months</th>
<th>2.5 months</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td></td>
<td>0 (b)**</td>
<td>2 (b)</td>
<td>40 (b)</td>
</tr>
<tr>
<td>2. Ca 2% extract</td>
<td></td>
<td>0 (b)</td>
<td>8 (b)</td>
<td>41 (b)</td>
</tr>
<tr>
<td>3. Pt 2% extract</td>
<td></td>
<td>2 (b)</td>
<td>12 (b)</td>
<td>38 (b)</td>
</tr>
<tr>
<td>4. Ca turned block***</td>
<td></td>
<td>0 (b)</td>
<td>10 (b)</td>
<td>36 (b)</td>
</tr>
<tr>
<td>5. Pt turned block</td>
<td></td>
<td>74 (a)</td>
<td>84 (a)</td>
<td>100 (a)</td>
</tr>
</tbody>
</table>

* Ca = *C. angustifolium*, Pt = *P. aquilinum.*
** The same letter in parentheses at the same column indicates not significantly different in Duncan's multiple test (95% c.l.). SEM = 2.87, 2.00, and 2.29 for 2 months, 2.5 months, and 3 months respectively, for conditions 2-5.
*** 8-month-old culture.

Table 9. Average antheridium formation (%) by *Campyloneurum angustifolium* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
<th>2 months</th>
<th>2.5 months</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td></td>
<td>0 (b)**</td>
<td>10 (c)</td>
<td>29 (c)</td>
</tr>
<tr>
<td>2. GA3 5x10⁻⁵ g/ml</td>
<td></td>
<td>0 (b)</td>
<td>6 (c)</td>
<td>19 (c)</td>
</tr>
<tr>
<td>3. Ca 10% extract</td>
<td></td>
<td>0 (b)</td>
<td>6 (c)</td>
<td>25 (c)</td>
</tr>
<tr>
<td>4. Pt 10% extract</td>
<td></td>
<td>0 (b)</td>
<td>9 (c)</td>
<td>30 (c)</td>
</tr>
<tr>
<td>5. Ca cleared block***</td>
<td></td>
<td>0 (b)</td>
<td>29 (b)</td>
<td>59 (b)</td>
</tr>
<tr>
<td>6. Ca inverted block***</td>
<td></td>
<td>0 (b)</td>
<td>42 (b)</td>
<td>58 (b)</td>
</tr>
<tr>
<td>7. Pt cleared block</td>
<td></td>
<td>85 (a)</td>
<td>94 (a)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>8. Pt inverted block</td>
<td></td>
<td>96 (a)</td>
<td>94 (a)</td>
<td>98 (a)</td>
</tr>
</tbody>
</table>

* Ca = *C. angustifolium*, Pt = *P. aquilinum.*
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 3.63, 7.86, and 10.63 for 2 months, 2.5 months, and 3 months respectively, for conditions 3-8.
*** 1-year-old culture.
Welling 1994). In this study, the genetic load was found to be low (average) for gametophytes of source "A", but very high (100) for those of "B". This suggests that sporophytes of "B" are from a highly outcrossing population, whereas those of "A" are derived from a population which originated through intragametophytic selfing. High levels of genetic load and intergametophytic mating have been found to characterize species of mature rain forests, whereas low levels of genetic load and intragametophytic selfing characterize pioneering species (Lloyd 1974a). Unfortunately, we do not know the natural origins of the two spore sources of *C. angustifolium* used in this study. We could not find natural populations in Florida although they may still survive in the Fakahatchee Strand State Preserve (Hammer pers. comm.) where *C. angustifolium* has been known historically. Therefore, we cannot verify the relation of habitats and mating systems here.

Masuyama (1979), Masuyama *et al.* (1987), and Masuyama and Watano (1990) demonstrated that diploid species favored intergametophytic mating whereas tetraploid species favored intragametophytic selfing. Both diploids, n = 37 (Evans 1963, originated from Peru), and tetraploids, n = 74 (Evans 1963, Costa Rica origin; Sorsa 1966, Costa Rica origin; Knobloch 1967, Jamaica origin) have been reported in *C. angustifolium*. It is possible that "A" sporophytes are tetraploid whereas "B" sporophytes are diploid.

Antheridiogen has been considered to be a mechanism promoting outcrossing (Peck *et al.* 1990; Haufler and Welling 1994). Secretions of living gametophytes by *P. aquilinum* and by 1-year-old *C. angustifolium* significantly promoted antheridium formation on *C. angustifolium* gametophytes. These results suggest that gametophytes of *C. angustifolium* produce and respond to their own antheridiogen (A_{can}) and respond to the antheridiogen of *P. aquilinum* (A_{pq}). There was no significant promotion of antheridium formation on blocks with 8-month-old *C. angustifolium* gametophytes, or when culture media extract of *C. angustifolium* or *P. aquilinum* gametophytes were added to the media (Tables 8, 9). This
perhaps is because the 8-month-old gametophytes are not mature enough to produce sufficient antheridiogen to induce antheridia. It is also possible that the gametophytes on the turned blocks are too few to produce enough antheridiogen. Supporting the latter is the observation that in multispore cultures (Table 1), antheridia appeared at 2.5 months. Similarly, the concentrations of the extracts are relatively low, compared to the media with living gametophytes, and thus the concentration in media with extract added may be not strong enough to facilitate antheridium formation. The failure of antheridium induction on C. angustifolium by their own extract could also be due to $A_{can}$ being short-lived or the extract method destroying its activity (Emigh and Farrar 1977). Because Onoclea sensibilis gametophytes are very sensitive to antheridiogen (Naf 1956), all the treatments produced early onset of antheridium formation (Tables 6, 7). This may be considered an additional evidence of the existence of antheridiogen in C. angustifolium and evidence that it is a stable compound. $A_{can}$ apparently is not similar to GA3 because the latter did not enhance antheridium formation in C. angustifolium. Furthermore, due to the response of C. angustifolium gametophytes to antheridiogen of P. aquilinum ($A_{pt}$), $A_{can}$ may be more closely related to $A_{pt}$.

Other indirect evidence of an antheridiogen system in C. angustifolium comes from the pattern of male expression in multi-spore cultures, where male gametophytes are smaller and antheridia are produced later than archegonia. This fits the model of antheridiogen function proposed by Naf (1963) who suggested that fast-growing females secrete antheridiogen which induces slow-growing gametophytes to produce antheridia.

In isolated-cultures, there is no significant difference in sexual expressions between gametophytes of isospore cultures and isogametophyte cultures. This suggests that the gametophytes have not produced antheridiogen by 1-month when the gametophytes were transferred to the iso-gametophyte cultures. In fact, the first male gametophytes appeared at
2.5 months after sowing spores in the multispore cultures. This seems to indicate that the antheridiogen begins to act between 1 month and 2.5 months. Although sexual expressions of gametophytes at the lowest (and darkest) plate layer were different from the other layers, all individuals at each layer were antheridium-bearing (either male or bisexual) at 8 months in culture. Antheridia on monospore-cultured gametophytes are likely induced by other factors, such as lobes formed by necrosis of intervening tissue (Haufler and Gastony 1978) or new-forming ameristic branches before antheridiogen sensitivity has been lost (Naf 1963).

Dopp (1950, cited from Naf 1963) found that antheridiogen-active extracts also retarded gametophyte growth except at very low concentration. In this experiment, similarly, all gametophytes growing in the treated cultures were smaller than those in untreated cultures (data not shown). In treated cultures, gametophytes derived from spores sown near other mature gametophytes (higher concentration of active substance) are smaller than those growing on medium containing extract (lower concentration of active substance). We here propose two possible reasons to explain the smaller gametophytes. One possibility is that the potential vegetative growth is diverted to antheridium production as demonstrated by Naf (1956). Although this can explain the stronger effect on small gametophytes growing near other mature gametophytes, it does not explain why asexual gametophytes growing in the treated conditions are of similar sizes to male gametophytes. Another possibility is that inhibitor substances are secreted from the mature gametophytes as shown in some species of Dryopteris (Bell 1958). To test this, we sowed spores of C. angustifolium on media treated as in conditions 5 and 6 in the table 9, but using mature gametophytes of Anemia phyllitidis instead of C. angustifolium as the antheridiogen source. A. phyllitidis is known to produce another type of antheridiogen (A_An) which induces antheridiogen production in Anemia but is inactive in families other than the Schizaeaceae (Naf et al. 1975). The size of C. angustifolium in these cultures remained very small, similar in size to the precocious male.
gametophytes described above, but all remained asexual during the 3-month culture (data not shown). These results indicate that inhibitors may be universal and more closely related among fern gametophytes, unlike antheridiogens which are more specific. It seems that *C. angustifolium* can produce its own antheridiogen as well as inhibitor, and respond to both of them as well as to those produced by *P. aquilinum*. On the other hand, for *A. phyllitidis*, *C. angustifolium* responds to its inhibitor but does not respond to the antheridiogen $A_A$. An alternative hypothesis is that antheridiogen functions in both antheridium promotion and gametophyte growth inhibition, but the efficiency of these two functions is different depending on the species. The statement that "antheridiogens actually affect size, and size influences sex expression" (Korpelainen 1994), is questioned here, at least in this species.

In the Polypodiaceae, neither antheridiogen presence nor response to $A_P$ has been reported (Voeller 1971; Raghavan 1989), except for a very weak response in *Aglamorpha meyeniana* (Naf 1966; 1969). However, Welling and Haufler (1993) demonstrated antheridiogen response in *Polypodium australe*. Haufler *et al.* (1995) found that the frequency of outcrossing was consistent with random mating in the *Polypodium vulgare* complex, a species (complex) thought to be unaffected by antheridiogen (Voeller 1964; Welling and Haufler 1993), and further pointed that this contradicts the viewpoint of "antheridiogen response and genetic load were coordinated in a predictable manner", stated by Schneller *et al.* (1990). In *C. angustifolium* (source A), the existence of an antheridiogen response reduces the probability of intragametophytic selfing of gametophytes by increasing the number of male plants. Here the antheridiogen system may compensate for some degree of inbreeding by facilitating occasional outcrossing to maintain genetic diversity, as pointed out in *Ceratopteris thalictroides* by Watano and Masuyama (1991). On the other hand, antheridiogen in polyploid *C. angustifolium* may be a vestige inherited from their diploid ancestors.
For source "A", isolated gametophytes derived from isolated spores are capable of producing the sporophytes through intragametophytic selfing, whereas gametophytes in populations developed from many spores can produce sporophytes through either intragametophytic selfing or intergametophytic mating since the antheridiogen may facilitate the production of many male gametophytes. Based on the laboratory demonstration of the correlation between antheridiogen response and genetic load (Schneller et al. 1990), gametophytes of source "B" very likely secrete antheridiogen too, due to their 100% genetic load, although we did not test this. Thus antheridiogen response, together with the clone-forming habits and prolonged life span, promote interaction between gametophytes and allow the "B" population to maintain a high genetic diversity.

Although the function was weak, some substance secreted from the mature gametophytes of *C. angustifolium* and *P. aquilinum* did substitute for the requirement of light for spore germination in the dark (Table 5). The concentration of the substance of 2% and 10% extract of *C. angustifolium* gametophyte cultures and 10% extract of *P. aquilinum* gametophyte cultures might be too low to substitute light requirement for spore germination of *C. angustifolium*. Similarly, fewer gametophytes on the turned block might not secrete enough substance to make spores of *C. angustifolium* germinate in darkness. The younger gametophyte age (8-month-old) of *C. angustifolium* for the extract and on the turned block may also explain the absence of response.

We do not know if this substance is the antheridiogen $A_{can}$ or the so-called germinin (Voeller 1971). In general, the functions of the substance for promoting antheridium formation and the substance for substituting a light requirement for spore germination are usually parallel (Voeller 1971), but Welling and Haufler (1993) hypothesized that these two substances may function independently. Our experiments do not directly test whether the two substances are the same chemical. Our results, on the one hand, show that the promotion for
antheridium formation and the substitution for light requirement of spore germination of *C. angustifolium* is higher by living gametophytes of *P. aquilinum* than by those of *C. angustifolium*, and is higher by living gametophytes of *C. angustifolium* than by their extract. These observations suggest that the substance operates in parallel for these two functions. On the other hand, a 10% extract of *P. aquilinum* cultures caused spores of *C. angustifolium* to germinate in darkness but did not promote antheridium formation on *C. angustifolium* gametophytes. Furthermore, among several effective treatments promoting spore germination of *C. angustifolium* in darkness, only gametophytes of *P. aquilinum* on inverted and cleared blocks induced antheridium formation on *C. angustifolium* (Table 5). These results suggest these two functions may operate independently. Without further evidence, we cannot determine whether the antheridiogen and the germinin of *C. angustifolium* are the same or different.

A soil spore bank has been demonstrated to exist for terrestrial species (Dyer and Lindsay 1992). For epiphytic species, fern spores are possibly deposited very deep in bark or bryophyte mat where light intensity may be not sufficient for spore germination. Antheridiogen (or germinin) may function in inducing germination of these "buried" spores. Through the antheridiogen influence, the resultant precocious gametophytes could produce antheridia and release active sperms to interact with older gametophytes. This model has been proposed previously (Voeller 1971; Schneller *et al.* 1990; Haufler and Welling 1994) for the terrestrial ferns, and may fit epiphytic species as well.

In summary, *Campyloneurum angustifolium*, an epiphytic fern of the New World, is able to survive perennially through branching and vegetative proliferations and produce antheridiogen which facilitates the establishment of male gametophytes. Sporophytes are produced through either outcrossing or inbreeding, depending on spore sources as evidenced by high or low level of genetic load respectively. Polyploidy may characterize the inbreeding
group, whereas the outcrossing group may be diploid. For the inbreeding group, the antheridiogen may function to alleviate low genetic diversity through increasing the number of male plants and thus the number of intergametophytic matings. Antheridiogen in polyploids may also be a vestige inherited from their diploid ancestors. Antheridiogen (germinin) also induces spores buried in humus or bryophyte mats to germinate. Antheridiogen (possibly plus germinin), together with a clone-forming habit, and prolonged life span, provides a mechanism to promote interaction and outcrossing with other gametophytes which may develop from spores either dispersed later or previously buried in the epiphytic substrate mat.

Acknowledgments

We thank Dr. Paul N. Hinz for help with statistical analysis, Marie Selby Botanical Garden for allowing collection spores, Dr. H. Luther and Dr. R. E. Rivero for assisting collection, Dr. Roger L. Hammer for providing information useful to collecting the materials, Mrs. Ming-Ren Huang for help with culture plants, data recording, and manuscript preparation. This research was supported by the Department of Botany, Iowa State University, and by the National Science Council of Taiwan, Republic of China.

Literature cited


2. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF CAMPYLONEURUM PHYLLITIDIS (L.) PRESL

This paper is prepared for publication in American Fern Journal

Wen-Liang Chiou¹, Donald R. Farrar¹, and Tom A. Ranker²

Introduction

The morphology of fern gametophytes has been studied and applied to taxonomy and systematics for a long time. Beyond the standard heart shapes depicted in most textbooks, great variations have also been shown (Atkinson 1973; Nayar and Kaur 1971). These variations include the ability to grow indeterminately and branch so that perennial gametophyte clones of considerable size may be produced (Farrar 1990; Dassler 1995). The morphological diversity may relate to the species' mating system and reproductive biology.

Four types of mating system in homosporous pteridophytes have been defined by Klekowski (1979): (1) intragametophytic selfing, (2) intergametophytic selfing, (3) intergametophytic crossing, and (4) intergametophytic mating. Among these mating systems, intragametophytic selfing has been previously suggested as being predominant in homosporous ferns due to the close proximity of antheridia and archegonia on bisexual gametophytes (Klekowski and Baker 1966; Klekowski 1973, 1979) and indeed there are some homosporous ferns which have very high rates of intragametophytic selfing (Crist and Farrar 1983; McCauley et al. 1985; Soltis and Soltis 1986a; Watano and Sahashi 1992). Polyploid species may also favor intragametophytic selfing due to duplicated alleles which mitigate the problem of recessive deleterious allele expression associated with selfing (Klekowski and Baker 1966; Lloyd 1974; Masuyama and Watano 1990). Recently, however, electrophoretic evidence has shown that intergametophytic mating is the most common

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breeding system in homosporous ferns (Haufler and Soltis 1984; Holsinger 1987; Soltis and Soltis 1986b; Soltis and Soltis 1990; Ranker 1992).

Several types of reproductive behavior can reduce the probability of intragametophytic selfing. Asynchronous maturation of male and female gametangia is one way to avoid intragametophytic selfing in bisexual gametophytes (Klekowski 1968, 1969; Masuyama 1974a, b). Possession of an antheridiogen system is another way to facilitate production of unisexual gametophytes, and thus promote intergametophytic mating (Naf et al. 1975; Voeller 1971; Hamilton and Lloyd 1991). Antheridiogen (or germinin, proposed by Voeller 1971) also overcomes the inhibition of spore germination in darkness (Voeller 1971; Schneller et al. 1990). However, except for Polypodium australe (Welleng and Haufler 1993), and Aglaomorpha meyeniana (Naf 1966; 1969), Polypodiaceae s.s. has been thought neither to produce its own antheridiogen nor to respond to the antheridiogen of Pteridium aquilinum (L.) Kuhn, the most common antheridiogen known (Voeller 1971; Raghavan 1989). Genetic load is also an obstacle to successful intragametophytic selfing, and the degree of genetic load has been used to measure sporophyte heterozygosity and probable breeding system (Lloyd 1974; Cousens 1988). However, most of the information known about the reproductive biology of ferns is from research on terrestrial ferns which encounter very different environmental events from epiphytic ferns.

Campyloneurum phyllitidis (L.) Presl, a member of the Polypodiaceae, distributed from Florida, Mexico, and the West Indies to Central and South America (Nauman 1993), is a common epiphytic species in South Florida (Lellinger, 1985). Neither the gametophyte morphology nor the reproductive biology of this species has been described. Due to the epiphytic habitat of this species and the nature of its bark and bryophyte substrate, dispersed spores are likely to be more widely separated, and the interaction of the subsequent gametophytes more hindered than in terrestrial species (Dassler 1995). Thus gametophytes of
epiphytic species might be more likely to produce their sporophytes through intragametophytic selfing. This paper investigates this issue in *C. phyllitidis*, addressing the following questions: (1) how are gametangia expressed in this species, (2) how much genetic load exists, (3) do gametophytes produce and/or respond to antheridiogen, (4) through which mating system are sporophytes produced, (5) how does gametophyte morphology relate to the reproductive system.

**Materials and methods**

Several different sources of *Campyloneurum phyllitidis* were used. Isozyme electrophoresis materials were collected from Castellow Hammock (CH), Jonathan Dickson State Park (JD), and Fakahatchee Strand State Preserve (FS) in southern Florida in August, 1994. Ten sporophytes were collected from each place. Because an insufficient amount of spores were collected from the field, we also used spores from two sporophytes planted in the greenhouse of the Department of Botany of Iowa State University for the gametophyte culture. "A" and "B" represented these two spore sources. Since antheridiogen of *Pteridium aquilinum* (L.) Kuhn (Apt) has been well studied (Naf *et al.* 1975), and gametophytes of *Onoclea sensibilis* L. have been used to "safely identify antheridium-inducing activity" due to their complete lack of spontaneous antheridium production in young stages (Naf 1956), these two species were also used in this study to assay the activity of antheridiogen of *Campyloneurum phyllitidis*. Spores of *Onoclea sensibilis* were collected from Marshall County, Iowa, in September, 1994 (*Chiou s.n.*), and those of *Pteridium aquilinum* were in part collected in June, 1991 from Iowa City, Iowa (*Farrar 91-6-26-27*), and in part were kindly sent by Dr. David Wagner, collected from Eugene, Oregon, in August 3, 1994.

Voucher specimens of *C. phyllitidis* were deposited in the Ada Hayden Herbarium (ISC) of the Department of Botany in Iowa State University. Spores obtained from fertile fronds (*Chiou and Farrar 1994*) were stored in the refrigerator at about 5°C.
Spores were sown on 1% agar-solidified media which contained Bold's macronutrients (Bold 1957) and Nitsch's micronutrients (Nitsch 1951), and a trace of ferric chloride (Peck 1985). Cultures were maintained under continuous, white fluorescent illumination of 200-3000 lux. Temperature was maintained between 20-24°C.

Gametophyte morphology was observed under dissecting and compound microscopes. Pictures were either taken by auto-camera or hand-drawn using a drawing tube. Sizes of gametophytes were measured as the width of the widest part. Gametophytes were observed every half month.

To harvest antheridiogen-enriched solution, the following procedure was used. One mg of spores of *C. phyllitidis* and *P. aquilinum* were separately sterilized with 10% Clorox for 5 minutes and then rinsed with sterilized water. The sterilized spores were then suspended in 2 ml of sterilized water. Two drops of the spore suspension were placed into each of several petri dishes of 8.5 cm diameter containing about 50 ml of agar media. The cultures of *C. phyllitidis* and *P. aquilinum* gametophytes were frozen at 8 months and 2 months old respectively, then thawed and the liquid extract collected. 2% and 10% dilutions of the extract were made by adding 20 drops (ca. 1 ml) of the extract into large petri dishes containing 50 ml of agar medium and the small petri dishes containing 10 ml of agar medium respectively. In addition to these extracts, GA3 (5x10^-5 g/ml) was also used to test antheridiogen response.

Considering the possibility that some antheridiogens might be short-lived (Emigh and Farrar 1977), that the time of harvesting extract might be not proper, or that the harvesting method may destroy antheridiogen activity, three additional experiments were conducted in which agar with mature growing gametophytes was used directly for the assay. In the first experiment, the agar with growing mature gametophytes was cut into 1 x 2 cm blocks. Then each agar block was turned on its side (turned block), so the gametophytes stood on one side.
of the agar block. Ten spores were sown on the top of each block and ten such blocks were
put into each of two petri dishes. In the second experiment, agar supporting mature
gametophytes was cut into four parts. Each part was placed upside down in another petri
dish, and spores were sown on the upside-down agar (inverted block). In the third
experiment, some gametophytes were removed from cultures to make a 1 cm wide lane
(cleared block), and spores were sown on these cleared areas (between mature
gametophytes). All gametophyte cultures assayed for presence of antheridiogen were 8
months old and 2 months old for *C. phyllitidis* and *P. pteridium* respectively.

The preparation of both extracts and blocks with growing gametophytes were used in
tests of germination capacity in darkness and promotion of antheridium formation in the
light. A complete randomized design was used. The percentage of plants forming antheridia
was counted as the number of male plus bisexual plants. Tests were repeated twice in the first
experiment (Tables 10, 12) and four times in the second and third experiments (Tables 11,
13). At each observation time, 25 gametophytes were removed from each dish in the first
experiment and 12 plants were removed from each dish in the second and third experiments.

Capability of spore germination in darkness and the promotion of spore germination
in the dark by antheridiogen were tested by wrapping two layers of aluminum foil around
spore-sown petri dishes. These wrapped dishes were placed in a dark cabinet, unwrapped and
examined one month later. Tests were repeated two (table 8) and three (table 9) times.
Germination percentage was determined by counting 100 spores. The proportion of male
plants was measured as the number of male gametophytes divided by the total number of
dark-germinated gametophytes. A complete randomized design was used.

Genetic load was calculated by comparing isolated-spore and isolated-gametophyte
cultures with paired-spore and paired-gametophyte cultures. These cultures were grown on
"jelly-mold" plates, each with 20 cells. Each cell contained about 6 ml of agar medium. In
each plate, a single spore was transferred onto each of 5 cells, and a one-month-old gametophyte which was still asexual was transferred onto each of another 5 cells. Another 10 cells were used as paired-spore and paired-gametophyte controls. Five such plates were separated by transparencies and stacked into transparent plastic vegetable crispers for a total of 50 replicates of each culture type. The light intensity of these cultures were maintained between 1500 lux (the bottom layer) to 3500 lux (the top layer). Plants were watered every two weeks after gametophytes were 4 months old. Sporophytes were determined to have been produced sexually by examination with a compound microscope. Genetic load was measured by counting the percentage of bisexual gametophytes failing to produce sporophytes. The five layers of plates were designated as blocks, and spore-culture vs. gametophyte-culture were designated as a split plot. Since two source of spores were used, a Latin Square was designed. Five treatments were "A", "B" (isolated-cultures of two different sources), "AA", "BB" (paired-cultures of two same sources), and "AB" (paired-cultures of different sources).

For isozyme observation, the grinding method and buffer followed Farrar (1990). Starch-gel electrophoresis and staining were conducted following Ranker et al. (1989). Eleven enzyme systems were scored, including aconitate hydratase (ACO), fructose-biphosphatase (FBP), isocitrate dehydrogenase (IDH), hexokinase (HK), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PGD), phosphoglucone isomerase (PGI), phosphoglucomutase (PGM), shikimate dehydrogenase (SkDH), and triosephosphate isomerase (TPI). To score the isozyme pattern, Werth's (1989) method was followed since C. phyllitidis has been reported as tetraploid (Evans 1963, Nauman 1993). The most anodal region of activity of each locus pair was given a numeric abbreviation of "1" (e.g. Hk-1). Members of a locus pair were abbreviated with letters. "a" designated to the more anodal region of activity (e.g. Hk-la vs. Hk-lb). Isozyme data were calculated and analyzed by BIOSYS-1 (Release 1.7; Swofford and Selander 1989).
Results

Morphology

In multispore cultures, the rhizoid was usually the first cell produced on germination (Figs. 1, 2) about 3 days after sowing spores. The basal cell underwent a second division (Fig. 3), resulting in a protonemal cell which subsequently produced a filament by serial divisions (Fig. 4). Occasionally, a second rhizoid appeared before the protonemal cell (Fig. 2). The length of the filament was usually two to six cells. In addition to the basal cell, rhizoids also emerged from cells of the filament other than the top cell. A longitudinal division usually occurred at the apical cell and/or the other cells of the filament (Figs. 5, 6). Subsequent divisions and expansion of filament cells formed a broad spathulate prothallus (Figs. 5, 7). An obconical meristematic cell was built at the anterior margin by two oblique divisions at the early or later spathulate stage of the prothallus (Figs. 5, 7, 8). Later, a pluricellular meristem replaced the single meristematic cell, and a symmetrical (or nearly) cordate gametophyte formed (Fig. 9). Occasionally, the pluricellular meristem occurred on the lateral side of the spathulate prothallus (Fig. 10). Eventually, a symmetric or near-symmetric cordate stage appeared (Figs. 9, 11, 13).

In addition to the margin, rhizoids also occurred on both dorsal and ventral sides of the midrib as well as on the wings, but on the latter rhizoids were fewer than on the midrib or on the margin.

After attaining a spathulate or cordate shape, gametophytes produced hairs. Unicellular, papillate, and secretory hairs appeared on the margin (Figs. 11-13, 15), occasionally on the surface, especially on older gametophytes (Figs. 11-12). Multicellular hairs (Figs. 16-18) frequently occurred on both dorsal and ventral sides of wings and midribs (Figs. 11-13), but only rarely on the margin of the gametophyte.
Figs. 1-18. Gametophyte development in *Campyloneurum phyllitidis*. Figs. 1-10. Germination and early development. Fig. 11. Female gametophyte. Fig. 12. Branched hermaphroditic gametophyte. Fig. 13. Branching female gametophyte. Fig. 14. Enlargement of the vegetative proliferation. Fig. 15. Marginal unicellular hair. Fig. 16. Marginal multicellular hair. Figs. 17, 18. Superficial multicellular hairs. An = antheridium, Ar = archegonium, Mh = multicellular hair, Uh = unicellular hair. Bar = 0.1 mm, unless otherwise indicated.
Archegonia, appearing at about 1.5 months, were distributed on a thickened midrib behind the meristem (Figs. 11, 12). At about 2 months, antheridia appeared, usually on the posterior part or sometimes near the apical meristem if a midrib had not formed (Fig. 12), or on the wings (Fig. 13). By this time most of the gametophytes had become female.

As gametophytes grew older, their wings became lobed and sections of the margin became active and produced additional meristems (Fig. 12). From these meristems, branches formed and produced additional meristems. Branches arising from one or a few cells also occurred (Figs. 13-14). Eventually a mat of overlapping branches formed (Fig. 37). Gametophytes continued growing over two years and several sporophytes were derived from many of the individual gametophytes, which still remained growing after sporophyte production (Fig. 38).

Sex sequence

In multispore cultures, gametangia did not appear until 1.5 months after sowing spores. Sexual frequency varied with the culture time (Table 1). Archegonia developed earliest and female plants remained most common during the culture of 3 months. Males and hermaphroditic plants appeared at 2 months. Males were relatively small (Table 1) and seemed to be developing from slowing individuals, perhaps from late-germinated spores. Hermaphrodites were relatively large (Table 1) and seemed more likely derived from female plants.

Among isolated-cultures, sex expression was not significantly different either between two sources or among different plate layers (data not shown). On the other hand, except for male plants, the sex expression was significantly different between the monospore and monogametophyte cultures, 8 months after spores were sown (Table 2). However, the total number of antheridium-bearing individuals was not significantly different (male+bisexual in table 2).
Figs. 19-30. Dark germination and antheridium formation in response treatments. Figs. 19-28. Campylopteleurum phyllitidis. Fig. 19. Dark-growing gametophyte with 1 antheridia derived from a spore germinated under darkness on a cleared block of Pteridium aquilinum gametophytes. Fig. 20. Dark-growing gametophyte with 3 antheridia derived from a spore germinated under darkness on an inverted block of P. aquilinum gametophytes. Fig. 21. Asexual dark-growing gametophyte growing on medium containing 10% extract of P. aquilinum gametophyte cultures. Fig. 22. Dark-growing gametophyte with 3 antheridia derived from a spore germinated under darkness on an inverted block of C. phyllitidis gametophytes. Fig. 23. 2 month old male gametophyte growing on an inverted block of P. aquilinum gametophytes. Fig. 24. 2 month old male gametophyte growing on a cleared block of P. aquilinum gametophytes. Fig. 25. 2 month old male gametophyte growing on a turned block of P. aquilinum gametophytes. Fig. 26. 2.5 month old male gametophyte growing on an inverted block of C. phyllitidis gametophytes. Fig. 27. 2.5 month old male gametophyte growing on a cleared block of C. phyllitidis gametophytes. Fig. 28. 2.5 month old male gametophyte growing on the media containing 10% extract of C. phyllitidis gametophyte cultures. Figs. 29-30. Onoclea sensibilis. Fig. 29. 0.5 month old gametophyte of O. sensibilis growing on an inverted block of C. phyllitidis. Fig. 30. 1.5 month old gametophyte of O. sensibilis growing on a cleared block of C. phyllitidis gametophytes. An = antheridium. Bar = 0.1 mm.
Figs. 31-36. Dark germination and antheridium formation by *Campyloneurum phyllitidis* in response to treatments. Fig. 31. From a dark-germinated spore on medium containing 10% extract of *P. aquilinum* gametophyte cultures. Fig. 32. From a dark-germinated spore sown on a cleared block of *P. aquilinum* gametophytes. Fig. 33. From a dark-germinated spore sown on a cleared block of *C. phyllitidis* gametophytes. Fig. 34. From a dark-germinated spore sown on an inverted agar block with *C. phyllitidis* gametophytes. Fig. 35. A 2 month old male gametophyte growing on an inverted agar block with *P. aquilinum* gametophytes. Fig. 36. A 2 month old male gametophyte growing on a cleared block with *C. phyllitidis* gametophytes. Bar = 0.1 mm.
Figs. 37-40. Gametophyte development and experimental response. Figs. 37-39. *Campyloneurum phyllitidis*. Fig. 37. One 6 month old clone-forming gametophyte. Fig. 38. A 2 year old clone-forming gametophyte with several sporophytes. Fig. 39. A small male gametophyte (arrow) growing beside a large female gametophyte. Fig. 40. A one month old gametophyte of *Onoclea sensibilis* growing beside gametophytes of *C. phyllitidis*. Bar = 1 cm for Fig. 38; bar = 1 mm for Figs. 37, 39; bar = 0.1 mm for Fig. 40.
Table 1. The sexual frequency (%) and gametophyte size of *C. phyllitidis* at different times in multi-spore cultures.

<table>
<thead>
<tr>
<th>Width (mm)</th>
<th>1.5 months</th>
<th>2 months</th>
<th>2.5 months</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A M F H*</td>
<td>A M F H</td>
<td>A M F H</td>
<td>A M F H</td>
</tr>
<tr>
<td>0.5</td>
<td>- - - -</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
</tr>
<tr>
<td>1</td>
<td>29 - - -</td>
<td>13 2 -</td>
<td>6 2 -</td>
<td>2 4 -</td>
</tr>
<tr>
<td>2</td>
<td>50 - - -</td>
<td>13 - 30 3</td>
<td>2 2 21 -</td>
<td>2 6 13 2</td>
</tr>
<tr>
<td>3</td>
<td>10 - 6 -</td>
<td>- - 17 4</td>
<td>- - 25 8</td>
<td>- - 19 13</td>
</tr>
<tr>
<td>&gt;=4</td>
<td>- - - -</td>
<td>- - 18 -</td>
<td>- - 27 7</td>
<td>- - 10 9</td>
</tr>
<tr>
<td>Total</td>
<td>94 - 6 -</td>
<td>26 2 65 7</td>
<td>8 4 73 15</td>
<td>4 10 67 24</td>
</tr>
</tbody>
</table>

* A = asexual, M = male, F = female, H = hermaphrodite.

Table 2. The sex expression (%) of *C. phyllitidis* in isolated-spore (spore) and isolated-gametophyte (gametophyte) cultures at 8 months after sowing spores.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Male^1</th>
<th>Female^2</th>
<th>Bisexual^3</th>
<th>Male+Bisexual^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore</td>
<td>17(a)</td>
<td>41(a)</td>
<td>42(b)</td>
<td>59(b)</td>
</tr>
<tr>
<td>Gametophyte</td>
<td>8(a)</td>
<td>11(b)</td>
<td>81(a)</td>
<td>89(a)</td>
</tr>
</tbody>
</table>

1, 2, 3, 4 The same letter in parentheses in the same column means no significant difference in Duncan's multiple test (95% c.l.). SEM = 5.65, 5.13, 4.21, and 5.78 for 1, 2, 3, and 4 respectively.

Genetic load

As shown in Table 3, on average, more sporophytes were produced in paired cultures than in isolated cultures. Among isolated cultures, the isolated-spore cultures yielded significantly fewer sporophytes than the isolated-gametophyte cultures. Similarly, the genetic load expression was significantly lower in the isolated-gametophyte cultures than in the isolated-spore cultures, but there was no significant differences between the two sources, either in the isolated-spore or isolated-gametophyte cultures (Table 3). Sporophyte production among all the paired-cultures and the isolated-gametophyte culture were not significantly different.
Table 3. The percentage of sporophyte production and genetic load (by surviving bisexual gametophytes) of 8-month-old *C. phyllitidis* in spore-cultures (spore) and gametophyte-cultures (gametophyte) of two sporophytes from the greenhouse.

<table>
<thead>
<tr>
<th>Source^1</th>
<th>% sporophyte</th>
<th>load</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spore^2</td>
<td>gametophyte^2</td>
</tr>
<tr>
<td></td>
<td>spore^4</td>
<td>gametophyte^4</td>
</tr>
<tr>
<td>A</td>
<td>9 (b)</td>
<td>40 (a)</td>
</tr>
<tr>
<td>AA</td>
<td>48 (a)</td>
<td>61 (a)</td>
</tr>
<tr>
<td>AB</td>
<td>55 (a)</td>
<td>64 (a)</td>
</tr>
<tr>
<td>BB</td>
<td>49 (a)</td>
<td>65 (a)</td>
</tr>
<tr>
<td>B</td>
<td>5 (b)</td>
<td>61 (a)</td>
</tr>
</tbody>
</table>

1 "A", "B", isolated culture; "AA", "AB", "BB", paired culture. "AA" & "BB" means paired gametophytes were from the same sporophyte, "AB" from two different sporophytes.

2, 3, 4, 5 The same letter in parentheses means no significant difference in Duncan's multiple test (95% c.l.). SEM = 8.45, 4.40, 7.21, and 6.17 for 2, 3, 4, and 5 respectively.

**Isozyme analysis**

Seventeen putative duplicated locus pairs were scored among the eleven enzyme systems. There was no variability within or among the three populations for 12 locus pairs. These were fixed for the same allele at four locus-pairs (Fbp-1a/b, Idh-a/b, Mdh-1a/b, and Tpi-1a/b) and for fixed interlocus heterozygosity at eight locus-pairs (Aco-a/b, Fbp-2a/b, Hk-a/b, Mdh-2a/b, 6Pgd-1a/b, Pgi-2a/b, Skd-a/b, Tpi-2a/b). The locus-pair Mdh-3a/b was fixed in a single allele in the samples from JD and FS population, Pgm-2a/b was fixed in all populations, but for a different allele at 2b in JD, whereas Pgm-3a/b was fixed in the samples from JD and CH population. The locus-pair 6Pgd-2a/b had fixed interlocus heterozygosity in populations JD and CH (Table 4).

The genetic similarity among the three populations was high in both Rogers' (1972) and Nei's (1978) genetic coefficient (Table 5). In the population JD, only one genotype was found at each of the locus-pairs; in the population CH, each of the two variable locus-pairs Lap-a/b Mdh-3a/b had two genotypes and combining to form three genotypes; in the
Table 4. Allele frequencies in three populations of *C. phyllitidis*. Sample sizes in each population are 10.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele</th>
<th>JD*</th>
<th>CH*</th>
<th>FS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aco-a</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Aco-b</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Fbp-1a</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Fbp-1b</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
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</tr>
<tr>
<td>Fbp-2a</td>
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<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Fbp-2b</td>
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<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Hk-a</td>
<td>1</td>
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<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Hk-b</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Idh-a</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Idh-b</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Lap-a</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Lap-b</td>
<td>2</td>
<td>1.00</td>
<td>0.10</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.90</td>
<td>0.35</td>
</tr>
<tr>
<td>Mdh-1a</td>
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<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Mdh-1b</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Mdh-2a</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Mdh-2b</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Mdh-3a</td>
<td>1</td>
<td></td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.00</td>
<td>0.90</td>
<td>1.00</td>
</tr>
<tr>
<td>Mdh-3b</td>
<td>1</td>
<td></td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.00</td>
<td>0.90</td>
<td>1.00</td>
</tr>
<tr>
<td>6pgd-1a</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>6pgd-1b</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>6pgd-2a</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td>0.60</td>
</tr>
<tr>
<td>6pgd-2b</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgi-2a</td>
<td>1</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Pgi-2b</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgm-2a</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgm-2b</td>
<td>1</td>
<td></td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pgm-3a</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>Pgm-3b</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>Skd-a</td>
<td>1</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Skd-b</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Tpi-1a</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Tpi-1b</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Tpi-2a</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Tpi-2b</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* JD = Jonathan Dickson State Park, CH = Castellow Hammock, FS = Fakahatchee Strand State Preserve.
Table 5. Matrix of Roger's genetic similarity (above diagonal) and Nei's unbiased genetic identity (below diagonal) coefficients between pairs of three populations.

<table>
<thead>
<tr>
<th>Population*</th>
<th>JD</th>
<th>CH</th>
<th>FS</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD</td>
<td></td>
<td>0.938</td>
<td>0.934</td>
</tr>
<tr>
<td>CH</td>
<td>0.946</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>FS</td>
<td>0.956</td>
<td>0.981</td>
<td>**</td>
</tr>
</tbody>
</table>

* JD = Jonathan Dickson State Park, CH = Castellow Hammock, FS = Fakahatchee Strand State Preserve.

population FS, each of the two variable locus-pairs Lap-a/b and Pgm-3a/b contained three genotypes and the variable locus-pair 6Pgd-2a/b contained 2 genotypes and combining to form six genotypes (Table 6). Since the genetic similarity of these three populations is so high, we combined them into one population (Table 7) and found that four locus-pairs (Lap-a/b and Pgm-3a/b) involved three genotypes, the other three locus-pairs (Mdh-3a/b, Pgm-2a/b, and 6Pgd-2a/b) involved two genotypes, these combined to form 10 genotypes in total.

**Spore germination in darkness**

Spores of *Campyloneurum phyllitidis* did not germinate in the dark or in treatments in which GA3, 2%, or 10% extractions of *C. phyllitidis* gametophyte cultures were added to the media, or when spores were sown on turned blocks with mature *C. phylliditis* gametophytes (Table 8, conditions 2, 3, 4; Table 9, conditions 2, 7, 9). Spores of *C. phyllitidis* did germinate in the dark in other treatment conditions (Tables 8, 9). Among these effective treatments, the secretion of living gametophytes of *Pteridium aquilinum* induced greater spore germination than either those of *C. phyllitidis* (Table 9, conditions 5, 6 vs. conditions 3, 4), or the extract of *P. aquilinum* gametophyte cultures (Table 9, conditions 5, 6 vs. condition 8). The dark germination rates of some treatments were indeed very low, i.e., 10% of *P. aquilinum* extract and the secretion of living gametophytes of *C. phyllitidis* (Table 9, conditions 3, 4, 8). Although not significantly different from the dark control, some spores
Table 6. Description of multilocus genotypes of two populations of *C. phyllitidis*.

<table>
<thead>
<tr>
<th>Locus</th>
<th>CH*</th>
<th>FS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lap-a/b</td>
<td>11/22</td>
<td>11/22</td>
</tr>
<tr>
<td>Mdh-3a/b</td>
<td>11/11</td>
<td>11/11</td>
</tr>
<tr>
<td>Pgm-3a/b</td>
<td>11/22</td>
<td>11/22</td>
</tr>
<tr>
<td>6Pgd-2a/b</td>
<td>11/11</td>
<td>11/11</td>
</tr>
<tr>
<td>Observed</td>
<td>10</td>
<td>30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Locus</th>
<th>CH*</th>
<th>FS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lap-a/b</td>
<td>11/22</td>
<td>11/22</td>
</tr>
<tr>
<td>Mdh-3a/b</td>
<td>11/11</td>
<td>11/11</td>
</tr>
<tr>
<td>Pgm-3a/b</td>
<td>11/22</td>
<td>11/22</td>
</tr>
<tr>
<td>6Pgd-2a/b</td>
<td>11/11</td>
<td>11/11</td>
</tr>
<tr>
<td>Observed</td>
<td>10</td>
<td>30</td>
</tr>
</tbody>
</table>

* CH = Castellow Hammock, FS = Fakahatchee Strand State Preserve.

Table 7. Description of multilocus genotypes and their percentage in a combination of three Florida populations of *C. phyllitidis*.

<table>
<thead>
<tr>
<th>Lap-a/b</th>
<th>Mdh-3a/b</th>
<th>Pgm-2a/b</th>
<th>Pgm-3a/b</th>
<th>6Pgd-2a/b</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>11/33</td>
<td>22/22</td>
<td>11/11</td>
<td>11/11</td>
<td>22/22</td>
<td>3.33</td>
</tr>
<tr>
<td>11/22</td>
<td>22/22</td>
<td>11/11</td>
<td>11/11</td>
<td>22/22</td>
<td>3.33</td>
</tr>
</tbody>
</table>

did germinate in those treatments whereas none did so in the dark controls.

Under the effective treatments, most dark-growing gametophytes produced antheridia (Figs. 19, 20, 22, 31-34), and there was no significant difference among the effective treatments (Tables 8, 9), although some remained asexual (Fig. 21). Dark germinated prothalli producing antheridia consisted of one to a few cells (Figs. 19-22). Active swimming sperms were released from the antheridia (Figs. 31-34).
Table 8. Average dark germination percentage and male percentage of *C. phyllitidis* at one month after sowing spores under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Germination %**</th>
<th>Male %**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. light control</td>
<td>92 (a)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>2. Dark control</td>
<td>0 (c)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>3. Cp 2% extract</td>
<td>0 (c)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>4. Cp turned block</td>
<td>0 (c)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>5. Pt 2% extract</td>
<td>6 (b)</td>
<td>83 (a)</td>
</tr>
<tr>
<td>6. Pt turned block</td>
<td>12 (b)</td>
<td>100 (a)</td>
</tr>
</tbody>
</table>

* Cp = *C. phyllitidis*, Pt = *P. aquilinum*.
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 1.25 and 1.26 for germination % and male % respectively, for conditions 3-6.

Table 9. Average dark germination percentage and male percentage of *C. phyllitidis* at one month after sowing spores under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Germination %**</th>
<th>Male %**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Light control</td>
<td>92 (a)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>2. Dark control</td>
<td>0 (c)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>3. Cp inverted block</td>
<td>2 (c)</td>
<td>67 (a)</td>
</tr>
<tr>
<td>4. Cp cleared block</td>
<td>6 (c)</td>
<td>72 (a)</td>
</tr>
<tr>
<td>5. Pt inverted block</td>
<td>14 (b)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>6. Pt cleared block</td>
<td>18 (b)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>7. Cp 10% extract</td>
<td>0 (c)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>8. Pt 10% extract</td>
<td>4 (c)</td>
<td>75 (a)</td>
</tr>
<tr>
<td>9. GA3 5x10^-5 g/ml</td>
<td>0 (c)</td>
<td>0 (b)</td>
</tr>
</tbody>
</table>

* Cp = *C. phyllitidis*, Pt = *P. aquilinum*.
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 1.94 and 12.88 for germination % and male % respectively, for conditions 3-8.

Antheridium promotion in *Onoclea sensibilis*

Antheridium formation by half-month old gametophytes of *Onoclea sensibilis* was significantly promoted when the spores were sown near the mature gametophytes of *C. phyllitidis*, and when 2% or 10% extract of *C. phyllitidis* gametophyte cultures were added to
the media (Tables 10, 11). After one month, a few of the gametophytes growing under the control condition produced antheridia but significantly less than those growing under treated conditions (Tables 10, 11). The influence of the extract (either 2% or 10%) was slightly less than or not significantly different from secretions from the living gametophytes (Tables 10, 11). Antheridia were produced from very young filaments or from spathulate through young

Table 10. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
<th>0.5 months</th>
<th>1 month</th>
<th>1.5 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>0 (c)**</td>
<td>2 (b)</td>
<td>10 (b)</td>
<td></td>
</tr>
<tr>
<td>2. Cp. 2% extract</td>
<td>32 (b)</td>
<td>82 (a)</td>
<td>86 (a)</td>
<td></td>
</tr>
<tr>
<td>3. Cp. turned block</td>
<td>86 (a)</td>
<td>100 (a)</td>
<td>100 (a)</td>
<td></td>
</tr>
</tbody>
</table>

* Cp = *C. phyllitidis*.
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 8.25, 7.07, and 4.24 for 0.5 months, 1 month, and 1.5 months respectively, for conditions 2 & 3.

Table 11. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
<th>0.5 months</th>
<th>1 month</th>
<th>1.5 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>0 (b)**</td>
<td>6 (c)</td>
<td>17 (c)</td>
<td></td>
</tr>
<tr>
<td>2. Cp 10% extract</td>
<td>50 (a)</td>
<td>79 (b)</td>
<td>77 (b)</td>
<td></td>
</tr>
<tr>
<td>3. Cp cleared block</td>
<td>73 (a)</td>
<td>92 (ab)</td>
<td>98 (a)</td>
<td></td>
</tr>
<tr>
<td>4. Cp inverted block</td>
<td>79 (a)</td>
<td>100 (a)</td>
<td>100 (a)</td>
<td></td>
</tr>
</tbody>
</table>

* Cp = *C. phyllitidis*.
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 14.41, 4.20, and 3.25 for 0.5 months, 1 month, and 1.5 months respectively, for conditions 2-4.
heart-shaped stages of prothallus development (Figs. 29, 30, 40). Usually only one or two antheridia were produced at the young stages but they were more abundant on the relatively mature gametophytes. Active sperms were released.

**Antheridium promotion in Campyloneurum phyllitidis**

All treatments except GA3 and 2% extract of *C. phyllitidis* and *P. aquilinum* gametophyte cultures significantly promoted antheridium formation 1.5 months after sowing spores (Tables 12, 13). The influence of *C. phyllitidis* gametophytes was less or non-significantly different in promoting antheridium onset in comparison to that of *P. aquilinum* (Tables 12, 13). The former usually promoted antheridium formation in the late spathulate or early cordate stages of gametophytes (Figs. 26-28, 36, 39), whereas the latter usually began to induce the antheridium formation when the gametophytes were still very young and at relatively small size (Figs. 23-25, 35). These antheridium-induced gametophytes released abundant active sperms (Figs. 35, 36), and always remained relatively small. Sperms released from 3-month-old gametophytes swam relatively sluggishly, and these gametophytes gradually became brown and died whereas the control gametophytes still grew well.

**Discussion**

The morphology and development of *Campyloneurum phyllitidis* gametophytes have not been previously described, but the gametophyte of *C. angustifolium* has been depicted as cordate and as Drynaria-type in development (Nayar 1962) or branched and clone-forming in older gametophytes (Chapter 1). In this observation, not only the Drynaria-type but also the Ceratopteris-type was found. Cordate gametophytes also become branched and clone forming in older ages. Clones formed by repeated branching and vegetative propagation may benefit this species by increasing the gametophyte's living space and prolonging its life span. These extended clonal and perennial gametophytes continuously form gametangia on their
Table 12. Average antheridium formation (%) by *Campyloneurum phyllitidis* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period 1.5 months</th>
<th>Period 2 months</th>
<th>Period 2.5 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>0 (c)**</td>
<td>8 (c)</td>
<td>8 (b)</td>
</tr>
<tr>
<td>2. Cp 2% extract</td>
<td>0 (c)</td>
<td>8 (c)</td>
<td>20 (b)</td>
</tr>
<tr>
<td>3. Pt 2% extract</td>
<td>0 (c)</td>
<td>0 (c)</td>
<td>8 (b)</td>
</tr>
<tr>
<td>4. Cp turned block</td>
<td>32 (b)</td>
<td>60 (b)</td>
<td>88 (a)</td>
</tr>
<tr>
<td>5. Pt turned block</td>
<td>52 (a)</td>
<td>100 (a)</td>
<td>100 (a)</td>
</tr>
</tbody>
</table>

* Cp = *C. phyllitidis*, Pt = *P. aquilinum*.  
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 2.83, 2.00, and 4.47 for 1.5 months, 2 months, and 2.5 months respectively, for conditions 2-5.

Table 13. Average antheridium formation (%) by *Campyloneurum phyllitidis* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period 1.5 months</th>
<th>Period 2 months</th>
<th>Period 2.5 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>0 (c)**</td>
<td>9 (c)</td>
<td>19 (c)</td>
</tr>
<tr>
<td>2. GA3 5x10^-5 g/ml</td>
<td>0 (c)</td>
<td>4 (c)</td>
<td>11 (c)</td>
</tr>
<tr>
<td>3. Cp 10% extract</td>
<td>28 (b)</td>
<td>51 (b)</td>
<td>65 (b)</td>
</tr>
<tr>
<td>4. Pt 10% extract</td>
<td>88 (a)</td>
<td>85 (a)</td>
<td>94 (a)</td>
</tr>
<tr>
<td>5. Cp cleared block</td>
<td>71 (a)</td>
<td>80 (a)</td>
<td>85 (a)</td>
</tr>
<tr>
<td>6. Cp inverted block</td>
<td>70 (a)</td>
<td>94 (a)</td>
<td>94 (a)</td>
</tr>
<tr>
<td>7. Pt cleared block</td>
<td>73 (a)</td>
<td>94 (a)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>8. Pt inverted block</td>
<td>84 (a)</td>
<td>92 (a)</td>
<td>100 (a)</td>
</tr>
</tbody>
</table>

* Cp = *C. phyllitidis*, Pt = *P. aquilinum*.  
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 11.85, 9.76, and 8.20 for 1.5 months, 2 months, and 2.5 months respectively, for conditions 3-8.
new proliferations, and thus enhance the possibility of interaction with other gametophytes which establish previously or later.

Intergametophytic mating has been recently thought to be the most common breeding system in homosporous ferns, and genetic load has been suggested to be the primary mechanism promoting outcrossing (Masuyama 1979, 1986; Haufler et al. 1990; Haufler and Welling 1994). In this study, the apparent genetic load was found to be higher in the isolated-spore cultures (average 79%) than that in the isolated-gametophyte cultures (average 35%). Gametophytes from two sources "A" and "B" produced similar results. The significantly lower sporophyte production in isolated-spore cultures may be due to absence of any gametophyte interaction (possible antheridiogen effect) whereas isolated gametophytes first germinated and grew in a population culture for one month. Most likely the latter promoted simultaneous maturation of male and female gametangia (Table 2 shows significantly fewer bisexual gametophytes in the isolated-spore cultures). Thus the higher apparent genetic load of isolated-spore cultures may be not due to recessive lethals, but more likely due to failure to obtain functional bisexuality.

The relatively high apparent genetic load suggests that sporophytes of *C. phyllitidis* are frequently produced by intergametophytic mating although intragametophytic selfing is possible. Pairing of gametophytes, either from the same or from different spore sources, failed to relieve the sporophyte suppression observed in isolated gametophyte cultures. This suggests that the genetic load is being expressed in gamete development (Klekowski 1970). If inviable eggs or non-functioning archegonia are being formed, pairing of gametophytes could be of no consequence.

Masuyama (1979), Masuyama et al. (1987), and Masuyama and Watano (1990) demonstrated that diploid species favored intergametophytic mating whereas tetraploid species favored intragametophytic selfing. A tetraploid chromosome number (n = 74, 2n =
148) has been reported in Florida populations of *C. phyllitidis* (Evans 1963, Naumen 1993). This may explain this species' ability to reproduce through to some extent intragametophytic selfing.

Strong evidence for the mating system of diploid species in the wild can be derived from isozyme electrophoretic patterns. However, electrophoretic patterns for *C. phyllitidis* revealed a high level of fixed interlocus heterozygosity, indicating that these samples from Florida are polyploid, probably allopolyploid. Because of this, accurate counts of heterozygous individuals and estimates of outcrossing are not possible, but at least one outcrossed individual (Lap-a$^{23}/b^{33}$) was among the 10 samples in the FS population.

Assuming allopolyploidy, the maximum number of genotypes per locus-pair indicates the minimum number of hybridization events involved in producing the species (Ranker *et al*. 1994). Thus the single genotype in the population JD suggests only one hybridization event occurred in the ancestry of this population in Jonathan Dickson State Park. Two genotypes at each of Lap-a/b and Mdh-3a/b of population CH indicate that the population originated from at least two hybridization events in Castellow Hammock. Three genotypes at each of Lap-a/b and Pgm-3a/b implies at least three hybridization events producing population in Fakahatchee Strand State Preserve (Table 6). On the other hand, the total number of multilocus genotypes estimates the number of hybridization events (Ranker *et al*. 1994), thus the total genotype number suggests that one, three, and six distinct hybridization events might have occurred in JD, CH, and FS respectively (Table 6), assuming that mutation and sexual recombination have played only a minor role. Because there are not great distances separating these populations in Florida and because of the high similarity of genotypes among the three populations, we may consider them as a single population. Thus the number of hybridization events responsible for the Florida plants is at least three and may be as high as ten. Because *C. phyllitidis* is widespread in tropical America and because likely
parental diploids are not present in Florida, these Florida genotypes likely represent three to ten separate spore introductions. The high level of genetic identity among the three Florida populations suggests that they may share a common and recent evolutionary history.

Antheridiogen has been considered to be one of the mechanisms promoting outcrossing (Haufler and Welling 1994). Since antheridium onset was significantly promoted both by culture extracts and living gametophytes of both *P. aquilinum* and *C. phyllitidis* (Tables 12, 13), *C. phyllitidis* likely produces and responds to its own antheridiogen (*A<sub>cp</sub>*<sub>h</sub>) as well as responds to the antheridiogen of *P. aquilinum* (*A<sub>p</sub>*<sub>t</sub>). The reason for no significant promotion of antheridium by 2% culture extracts of *C. phyllitidis* and *P. aquilinum* gametophytes may be due to an insufficient concentration of the antheridiogen. These treatments did promote antheridium formation on the gametophytes of *Onoclea sensibilis* (Tables 10, 11) which are more sensitive to antheridiogen. *A<sub>cp</sub>*<sub>h</sub> apparently is not similar to GA3 because the latter did not enhance antheridium formation in *C. phyllitidis*. Furthermore, since *C. phyllitidis* responds to antheridiogen of *P. aquilinum* (*A<sub>p</sub>*<sub>t</sub>), *A<sub>cp</sub>*<sub>h</sub> may be similar to *A<sub>p</sub>*<sub>t</sub>.

Other indirect evidence of an antheridiogen system in *C. phyllitidis* comes from the pattern of male expression in multi-spore cultures, where male gametophytes were smaller and antheridia were produced later than archegonia (Table 1). This fits the model of antheridiogen function proposed by Naf (1963) who suggested that fast-growing females secrete antheridiogen which induces slow-growing gametophytes to produce antheridia.

In isolated-cultures, there was a significant difference between gametophytes of the monospore cultures and monogametophyte cultures. This may reflect effective antheridiogen secretion before the gametophytes were transferred at 1-month-old. Therefore more monospore-culture gametophytes were female, since no other gametophytes produced antheridiogen to affect them. Antheridia on some monospore-cultured gametophytes are
likely induced by other factors, such as lobes formed by necrosis of intervening tissue (Haufler and Gastony 1978) or new-forming ameristic branches before antheridiogen sensitivity has been lost (Naf 1963).

Dopp (1950, cited from Naf 1963) found that antheridium extracts also retarded gametophyte growth except at very low concentration. In this experiment, similarly, all gametophytes growing in the treated cultures were smaller than those in untreated cultures (data not shown). In treated cultures, gametophytes derived from those spores sown near other mature gametophytes (higher concentration of active substance) were smaller than those growing on medium containing extract (lower concentration of active substance). We here propose two possible reasons to explain the smaller gametophytes. One possibility is that the potential vegetative growth is diverted to antheridium production as demonstrated by Naf (1956). Although this can explain the stronger effect on small gametophytes growing near other mature gametophytes, it does not explain why asexual gametophytes growing in the treated conditions are of similar sizes to male gametophytes. Another possibility is that an inhibitor substance is secreted from the mature gametophytes as shown in some species of Dryopteris (Bell 1958). To test this, we sowed spores of C. phyllitidis on media treated as in conditions 5 and 6 in the table 13, but using mature gametophytes of Anemia phyllitidis instead of C. phyllitidis as the antheridiogen source. A. phyllitidis is known to produce another type of antheridiogen (A\textsubscript{AN}) which induces antheridium production in Anemia but is inactive in families other than the Schizaeaceae (Naf et al. 1975). The size of C. phyllitidis in these cultures remained very small, similar in size to the precocious male gametophytes described above, but all remain asexual during the 3-month culture (data not shown). This result indicates that inhibitors may be universal and closely related among fern gametophytes, unlike antheridiogens which are more specific. It seems that C. phyllitidis can produce its own antheridiogen as well as inhibitor, and respond to both of them as well as to
those produced by *P. aquilinum*. On the other hand, for *A. phyllitis*, *C. phyllitis* responds to its inhibitor but not to its antheridiogen $A_{An}$. An alternative hypothesis is that antheridiogen functions in both antheridium promotion and gametophyte growth inhibition, but the efficiency of these two functions is different depending on the species. The statement that "antheridiogens actually affect size, and size influences sex expression" (Korpelainen 1994), is questioned here, at least in this species.

In the Polypodiaceae, neither antheridiogen presence nor response to $A_{pt}$ has been thought to exist (Voeller 1971; Raghavan 1989), except for a very weak response in *Aglamorpha meyeniana* (Naf 1966; 1969). However, Welling and Haufler (1993) recently demonstrated that gametophytes of *Polypodium australe* produce antheridiogen. Haufler et al. (1995) found that the frequency of outcrossing was consistent with random mating in the *Polypodium vulgare* complex, a species (complex) thought to be unaffected by antheridiogen (Voeller 1964; Welling and Haufler 1993), and further pointed that this contradicts the viewpoint that "antheridiogen response and genetic load were coordinated in a predictable manner", stated by Schneller et al. (1990). In *C. phyllitis*, the existence of an antheridiogen response may reduce the probability of intragametophytic selfing of gametophytes by increasing the number of male plants.

Some substance secreted from mature gametophytes of *C. phyllitis* and *P. aquilinum* promotes spores of *C. phyllitis* to germinate in darkness, substituting for the requirement of light for spore germination (Tables 8, 9). We do not know if this substance is the antheridiogen $A_{cph}$ or the so-called germinin (Voeller 1971). In general, the functions of the substance for promoting antheridium formation and the substance substituting for a light requirement for spore germination are parallel (Voeller 1971), but Welling and Haufler (1993) hypothesized that these two substances may function independently. In this experiment, all treatments, except for the culture extract of *C. phyllitis*, functioned alike in
promoting antheridium production and spore dark germination of *C. phyllitidis*. The 10% extract promoted antheridium production but not dark germination. It is possible that antheridiogen is different from germinin and that the latter is short-lived or loses its activity in the extraction process, and thus extracts contained no active germinin. It is also possible that germinin and antheridiogen are the same thing, but spore dark germination of this species requires a higher concentration of antheridiogen (germinin). The fact that gametophytes of *C. phyllitidis* on turned blocks did not promote spore dark germination but did hasten antheridium production may support the latter hypothesis. In addition, the fact that extracts of *P. aquilinum* gametophyte cultures promoted spore dark germination suggests that germinin was not destroyed by extraction (at least in *P. aquilinum*) and supports different required concentrations for spore dark germination and for antheridium promotion. Without more direct evidence, we can not determine whether the two substances are the same chemical.

A soil spore bank has been demonstrated to exist for terrestrial species (Dyer and Lindsay 1992). For epiphytic species, fern spores are possibly deposited very deep in bryophyte mats where light intensity may be insufficient for spore germination. Antheridiogen (or germinin) may function in inducing germination of these "buried" spores. Through the antheridiogen influence, the resultant gametophytes could precociously produce antheridia and release active sperms which could interact with older gametophytes. This model has been proposed previously (Voeller 1971; Schneller *et al.* 1990; Haufler and Welling 1994) for terrestrial ferns, and may fit epiphytic species as well.

In summary, gametophytes of *Campyloneurum phyllitidis*, an epiphytic fern of the New World, are able to survive perennially through branching and clone formation. Sporophytes are produced through both intragametophytic selfing and intergametophytic mating, but the proportion of each is difficult to assess. Multiple bands and fixed
heterozygosity at most enzyme loci indicate polyploidy which may favor intragametophytic selfing, but fairly high genetic load implies frequent outcrossing. Gametophyte interaction (via antheridiogen) temporarily occurring at early gametophyte stages promotes successful intragametophytic selfing by increasing bisexual development. At the same time, a relatively persistent antheridiogen influence increases the number of male plants and thus the opportunity for intergametophytic matings. Antheridiogen, or germinin, also induces spores buried in humus or bryophyte mats to germinate. Antheridiogen (plus germinin?), together with a clone-forming habit and prolonged life span promote interaction with other gametophytes which may develop from spores either dispersed later or previously buried in the epiphyte substrate. Outcrossing in its diploid ancestors is implied by the multiple independent hybridization events needed to produce the genotypic variability observed in C. Phyllitisidis.

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Literature cited


3. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF *LEPISORUS THUNBERGIANUS* (KAULF.) CHING

This paper is prepared for publication in American Fern Journal

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**Introduction**

The morphology of fern gametophytes has been studied and applied to taxonomy and systematics for a long time. Beyond the standard heart shapes depicted in most textbooks, great variations have also been shown (Atkinson 1973; Nayar and Kaur 1971). These variations include the ability to grow indeterminately and to branch so that perennial gametophyte clones of considerable size may be produced (Farrar 1990; Dassler 1995). The morphological diversity may relate to the species' mating system and reproductive biology.

Four types of mating system in homosporous pteridophytes have been defined by Klekowski (1979): (1) intragametophytic selfing, (2) intergametophytic selfing, (3) intergametophytic crossing, and (4) intergametophytic mating. Among these mating systems, intragametophytic selfing has been previously suggested as being predominant in homosporous ferns due to the close proximity of antheridia and archegonia on bisexual gametophytes (Klekowski and Baker 1966; Klekowski 1973, 1979) and indeed there are some homosporous ferns which have very high rates of intragametophytic selfing (Crist and Farrar 1983; McCauley et al. 1985; Soltis and Soltis 1986a; Watano and Sahashi 1992). Polyploid species may also favor intragametophytic selfing due to duplicated alleles which mitigate the problem of recessive deleterious allele expression associated with selfing (Klekowski and Baker 1966; Lloyd 1974; Masuyama and Watano 1990). Recently, however, electrophoretic evidence has shown that intergametophytic mating is the most common

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Several types of reproductive behavior can reduce the probability of intragametophytic selfing. Asynchronous maturation of male and female gametangia is one way to avoid intragametophytic selfing in bisexual gametophytes (Klekowski 1968, 1969; Masuyama 1974a, b). Possession of an antheridiogen system is another way to facilitate production of unisexual gametophytes, and thus promote intergametophytic mating (Naf et al. 1975; Voeller 1971; Hamilton and Lloyd 1991). Antheridiogen (or germinin, proposed by Voeller 1971) also overcomes the inhibition of spore germination in darkness (Voeller 1971; Schneller et al. 1990). However, except for Polypodium australe (Welling and Haufler 1993), and Aglaomorpha meyeniana (Naf 1966; 1969), Polypodiaceae s.s. has been thought neither to produce its own antheridiogen nor to respond to the antheridiogen of Pteridium aquilinum (L.) Kuhn, the most common antheridiogen known (Voeller 1971; Raghavan 1989). Genetic load is also an obstacle to successful intragametophytic selfing, and the degree of genetic load has been used to measure sporophyte heterozygosity and probable breeding system (Lloyd 1974; Cousens 1988). However, most of the information known about the reproductive biology of ferns is from research on terrestrial ferns which encounter very different environmental events from epiphytic ferns.

Lepisorus thunbergianus, a member of the Polypodiaceae, is an epiphytic species that occurs in S. and E. China, Taiwan, Japan, Philippines (DeVol and Kuo 1975), and also in Hawaii. Due to the epiphytic habitat of this species and the nature of its bark and bryophyte substrate, the effective distance between dispersed spores is increased, so that the interaction of the subsequent gametophytes is hindered. Thus, gametophytes of this species would seem more likely to produce their sporophytes through intragametophytic selfing. This paper will
address the following questions: (1) through which mating system are sporophytes produced, (2) how are gametangia expressed, (3) do gametophytes produce and/or respond to antheridiogen, (4) how much genetic load exists, (5) how is their morphology related to the reproductive system.

Materials and methods

Spores of *Lepisorus thunbergianus* were collected from Hawaii in August, 1992 (*Farrar 92-8-16-4*). Since antheridiogen of *Pteridium aquilinum* (*AP*) has been studied extensively (Naf *et al.* 1975), and gametophytes of *Onoclea sensibilis* L. have been used to "safely identify antheridium-inducing activity" due to their complete lack of spontaneous antheridium production in early stages (Naf 1956), these two species were also used in this study to assay the activity of antheridiogen of *L. thunbergianus*. Spores of *Onoclea sensibilis* were collected from Marshall County, Iowa, in September, 1994 (*Chiou s.n.*), and those of *Pteridium aquilinum* were collected in June, 1991 from Iowa City, Iowa (*Farrar 91-6-26-27*), and also from Eugene, Oregon, in August 3, 1994 (courtesy of Dr. David Wagner). Voucher specimens of *L. thunbergianus* were deposited in the Ada Hayden Herbarium (ISC) of the Department of Botany at Iowa State University. Spores were obtained from fertile fronds (*Chiou and Farrar 1994*) and stored in a refrigerator at about 5°C.

Spores were sown on 1% agar-solidified media which contained Bold's macronutrients (Bold 1957) and Nitsch's micronutrients (Nitsch 1951), and a trace of ferric chloride (Peck 1985). Cultures were maintained under continuous, white fluorescent illumination of 2000-3000 lux. Temperature was maintained between 20-24°C.

Gametophyte morphology was observed under dissecting and compound microscopes. Pictures were taken either by auto-camera or hand-drawn using a drawing tube. Sizes of gametophytes were measured as the width of the widest part. Gametophytes were observed every half month.
To harvest antheridiogen-enriched solution, the following procedure was used. One mg of spores of *L. thunbergiana* and *P. aquilinum* were separately sterilized with 10% Clorox for 5 minutes and then rinsed with sterilized water. The sterilized spores were then suspended in 2 ml of sterilized water. Two drops of the spore suspension were placed into each of several petri dishes of 8.5 cm diameter containing about 50 ml of agar media. The cultures of *L. thunbergiana* and *P. aquilinum* gametophytes were frozen at 8 months and 2 months old respectively, then thawed and the liquid extract collected. 2% and 10% dilutions of the extract were made by adding 20 drops (ca. 1 ml) of the extract into large petri dishes containing 50 ml of agar medium and small petri dishes containing 10 ml of agar medium respectively. In addition to these extracts, GA3 (5x10^-5 g/ml) was also used to test antheridiogen response.

Considering the possibility that some antheridiogens might be short-lived (Emigh and Farrar 1977), that the time of harvesting extract might be not proper, or that the harvesting method may destroy antheridiogen activity, three additional experiments were conducted in which agar with mature growing gametophytes was used directly for the assay. In the first experiment, the agar with growing mature gametophytes was cut into 1 x 2 cm blocks. Then each agar block was turned on its side (turned block), so the gametophytes stood on one side of the agar block. Ten spores were sown on the top of each block and ten such blocks were put into each of two petri dishes. In the second experiment, agar supporting mature gametophytes was cut into four parts. Each part was placed upside down in another petri dish, and spores were sown on the upside-down agar (inverted block). In the third experiment, some gametophytes were removed from cultures to make a 1 cm width lane (cleared block), and spores were sown on these cleared areas (between mature gametophytes). All gametophyte cultures assayed for presence of antheridiogen were 8 months and 2 months old for *L. thunbergiana* and *P. aquilinum* respectively.
Preparations of both extracts and blocks with growing gametophytes were used in tests of germination in darkness and promotion of antheridium formation in the light. A complete randomized design was used. The percentage of plants forming antheridia was counted as the number of male plus bisexual individuals. Tests were repeated twice in the first experiment (Tables 7, 9) and four times in the second and third experiments (Tables 8, 10). At each observation time, 25 gametophytes were removed from each dish in the first experiment and 12 gametophytes from each dish in the second and third experiments.

Capability of spore germination in darkness and the promotion of spore germination in the dark by antheridiogen were tested by wrapping two layers of aluminum foil around spore-sown petri dishes. These wrapped dishes were placed in a dark cabinet, and unwrapped and examined one month later. Tests were repeated two (table 5) and three (table 6) times. Germination rates were determined by counting 100 spores. The proportion of male plants was measured as the number of male gametophytes divided by the total number of dark-germinated gametophytes. A complete randomized design was used.

Genetic load was calculated by comparing isolated-spore and isolated-gametophyte cultures with paired-spore and paired-gametophyte cultures. These cultures were grown on "jelly-mold" plates, each with 20 cells. Each cell contained about 6 ml of agar medium. In each plate, a single spore was transferred onto each of 5 cells, and a one-month-old gametophyte which was still asexual was transferred onto each of another 5 cells. Another 10 cells were used as paired-spore and paired-gametophyte controls. Five such plates were separated by transparencies and stacked into transparent plastic vegetable crispers for a total of 50 replicates of each culture type. The light intensity of these cultures were maintained between 1500 lux (the bottom layer) to 3500 lux (the top layer). Plants were watered every two weeks after gametophytes were 4 months old. Sporophytes were determined to have been produced sexually by examination with a compound microscope. Genetic load was measured
by counting the percentage of bisexual gametophytes failing to produce sporophytes. The five layers of plates were designated as blocks, and spore-cultures vs. gametophyte-cultures were designated as a split plot.

For isozyme observation, 40 individuals were collected by D. R. F. in 1992 and 1993 from Hawaii. The grinding method and buffer followed Farrar (1990). Starch-gel electrophoresis and staining techniques followed Ranker et al. (1989). Twelve enzyme systems were scored, including aconitate hydratase (ACO), adolase (ALD), fructose-biphosphatase (FBP), glutamate oxaloacetate transaminase (GOT), hexokinase (HK), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PGD), phosphoglucose isomerase (PGI), phosphoglucomutase (PGM), shikimate dehydrogenase (SkDH), and triosephosphate isomerase (TPI). To score the isozyme pattern, following Werth (1989), the most anodal region of activity of each locus pair was given a numeric abbreviation of "1" (e.g. Hk-1). Members of a locus pair were abbreviated with letters, "a" assigned to the more anodal region of activity (e.g. Hk-1a vs. Hk-1b). Isozyme data were calculated and analyzed by BIOSYS-1 (Release 1.7; Swofford and Selander 1989).

Results

Morphology

In multispore cultures, spores began to germinate one week after sowing the spores. Viability of spores remained through 1.5 years of storage in the refrigerator at 5°C, but the germination rate declined to 62% and the beginning of germination was delayed to about two weeks when spores were stored for more than two years. After the basal cell, the rhizoid was usually the first cell produced in germination (Fig. 1). The basal cell underwent a second division (Fig. 2), resulting in a protonemal cell which subsequently produced a filament by serial divisions (Fig. 3). The lengths of filaments varied from two to six cells. In addition to the basal cell, rhizoids also emerged from other cells of the filament below the top cell.
Figs. 1-17. Gametophyte development and gametophyte response to experimental treatments. Figs. 1-15. *Lepisorus thunbergianus*. Figs. 1-8. Germination and early development. Figs. 9-11. Female gametophytes. Figs. 12, 13. Hermaphroditic gametophytes. Fig. 14. A male gametophyte. Fig. 15. 1.5 month old gametophyte growing on a cleared block of mature *L. thunbergianus* gametophyte. Figs. 16-17. *Onoclea sensibilis*. Fig. 16. 0.5 month old gametophyte of *O. sensibilis* growing on medium containing 2% extract of mature *L. thunbergianus* gametophyte cultures. Fig. 17. 1 month old gametophyte of *O. sensibilis* growing on medium containing 10% of extract of mature *L. thunbergianus* gametophyte cultures. Bar = 0.1 mm unless otherwise indicated.
About one week after germination, a longitudinal division usually occurred in the apical cell and/or the cells below (Figs. 4, 5), and a spathulate prothallus resulted (Fig. 7). Later an obconical meristematic cell was built at the anterior margin by two oblique divisions later (Fig. 6). A pluricellular meristem replaced the single meristematic cell later (Fig. 8), and a nearly symmetrical cordate gametophyte formed eventually (Figs. 10, 12). The cordate gametophyte might be broad (Fig. 10) or narrow and elongated (Fig. 9).

In addition to the margin, rhizoids also occurred on both sides of the midrib as well as on the wings, but those on the latter were less frequent than those on the midrib or on the margin. Rhizoids even emerged from archegonia (Fig. 20). Clusters of marginal rhizoids sometimes appeared, especially on relatively elongated gametophytes (Figs. 9, 11).

After attaining spathulate stage, gametophytes produced hairs (Figs. 6, 7), most of which were unicellular, papillate, and secretory. These unicellular hairs first appeared on the margins, then on both dorsal and ventral sides of wings and midribs, but the frequencies were various. Hairs on the margin were very few (Fig. 10) to relatively common (fig. 13). Surface hairs were absent in some gametophytes (Figs. 10, 11).

At about 1.5 months, archegonia appeared and were distributed on the midrib behind the meristem (Figs. 9-13). Antheridia appeared at about 2 months usually on the posterior part (Fig. 12), or sometimes located near the apical meristem if the midrib had not formed (Fig. 14). Antheridia and archegonia were found either on the ventral or the dorsal side or both. Archegonia were occasionally located discontinuously along the midrib (Figs. 11, 19). On the older gametophytes, antheridia usually mixed with archegonia (Figs. 13, 21).

As gametophytes grew older, meristems often divided forming dichotomous branches (Figs. 9, 11). Branches formed repeatedly and frequently overlapped. Branches arising from one or a few cells also occurred. A mat of overlapping branches thus formed (Fig. 18). Gametangia formed continuously on the new branches. Gametophytes continued to grow for
Figs. 18-23. Gametophyte morphology of *Lepisorus thunbergianus* and response to experimental treatments. Figs. 18-21. Gametophyte morphology. Fig. 18. A clone of *L. thunbergianus* growing from a single spore. Fig. 19. Discontinuous midrib and archegonia. Fig. 20. A rhizoid from an archegonium. Fig. 21. Archegonia (long arrow) growing mixed with antheridia (short arrow). Figs. 22-23. Spore germination in darkness. Fig. 22. A dark-growing gametophyte on media containing 10% extract of *P. aquilinum* gametophyte cultures. Fig. 23. A dark-growing gametophyte on a cleared block of mature *P. aquilinum* gametophytes. Bar = 1 cm for Fig. 18; bar = 0.1 mm for Figs. 19-23.
over two years and several sporophytes could be derived from one single gametophyte which still remained growing after sporophyte production.

**Sex sequence**

In multispore cultures, gametangia did not appear until 1.5 months after sowing spores (Table 1). Archegonia appeared first and female plants remained most frequent throughout the 3 months of culture. Males and the hermaphrodites appeared at 2 months. The former remained relatively less frequent during the 3 months of culture (Table 1). Male gametophytes were relatively small in size and seemed to develop from slow growing gametophytes, perhaps late-germinating spores. Hermaphrodites gradually became the principal form after 4 months, and all the gametophytes became bisexual eventually (data not shown). Hermaphrodites were very likely derived from female gametophytes due to their relatively large size (Table 1; Fig. 13), but some relatively smaller hermaphrodites (Fig. 12) might have been derived from male gametophytes.

Among isolated-cultures, sex expression percentages were not significantly different either among different plate layers or between the spore- and gametophyte-culture (data not shown). All the gametophytes became hermaphroditic eventually.

**Genetic load**

As shown in table 2, only a few sporophytes appeared in both isolated-spore and isolated-gametophyte cultures at 8 months after sowing spores. Thus the genetic load was very high, up to 95% on average, and there was no significant difference between the spore-cultures and gametophyte-cultures. Paired-cultures were relatively more successful in producing syngamous sporophytes (22% on average), and there was no significant difference between the two types of paired-cultures (Table 2). However, in the paired-cultures, production of sporophytes was still low, only four to five times that of the isolated-cultures. Sporophytes yielded from the isolated-culture grew as well as those from the paired-
Table 1. The sexual frequency (%) and gametophyte size of *L. thunbergianus* at different ages in multi-spore cultures.

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<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>-</td>
<td>23</td>
<td>-</td>
</tr>
</tbody>
</table>

* A = asexual, M = male, F = female, H = hermaphrodite.

Table 2. The percentage of sporophyte production and genetic load of 8 month old *L. thunbergianus* in spore-cultures (spore) and gametophyte-culture (gametophyte).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% sporophyte</th>
<th>load</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spor gametophyte average</td>
<td>spor gametophyte average</td>
</tr>
<tr>
<td>isolated</td>
<td>6(b)^1</td>
<td>94(a)^3</td>
</tr>
<tr>
<td>paired</td>
<td>21(a)^1</td>
<td>96(a)^3</td>
</tr>
<tr>
<td></td>
<td>4(b)^1</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>23(a)^1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>22(a)^2</td>
<td>-</td>
</tr>
</tbody>
</table>

1, 2, 3 The same letter in parentheses means no significant difference in Duncan's multiple test (95% c.l.). SEM = 4.49, 3.12, and 2.08 for 1, 2, and 3 respectively.

Sporophyte production was not significantly different among different plate layers (data not shown).

**Isozymes**

Nineteen putative duplicated locus pairs were scored among the twelve enzyme systems (Table 3). A single allele was fixed at each of eight locus-pairs: Ald-a/b, Fbp-a/b, Got-1a/b, Mdh-1a/b, 6Pgd-2a/b, Pgi-1a/b, Pgm-1a/b, Tpi-1a/b. Among the remaining 11 locus-pairs, nine of them (Aco-a/b, Got-2a/b, Mdh-2a/b, Mdh-3a/b, 6Pgd-1a/b, Pgi-2a/b,
Table 3. Allele frequencies in populations of *L. thunbergianus*. The sample size is 40.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aco-a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Aco-b</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>Ald-a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Ald-b</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Fbp-a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Fbp-b</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Got-1a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Got-1b</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Got-2a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Got-2b</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>Hk-a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Hk-b</td>
<td>2</td>
<td>0.96</td>
</tr>
<tr>
<td>Lap-a</td>
<td>1</td>
<td>0.48</td>
</tr>
<tr>
<td>Lap-b</td>
<td>3</td>
<td>1.00</td>
</tr>
<tr>
<td>Mdh-1a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Mdh-1b</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Mdh-2a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Mdh-2b</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>Mdh-3a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Mdh-3b</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>6Pgd-1a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>6Pgd-1b</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>6Pgd-2a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>6Pgd-2b</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgi-1a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgi-1b</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgi-2a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgi-2b</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgm-1a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgm-1b</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgm-2a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgm-2b</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>Skd-a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Skd-b</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>Tpi-1a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Tpi-1b</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Tpi-2a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Tpi-2b</td>
<td>2</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Pgm-2a/b, Skd-a/b, Tpi-2a/b) showed fixed interlocus heterozygosity. A few sporophytes showed variation in the alleles of loci Hk-a/b and Lap-a/b. One of the variable locus pairs (Hk-a/b) contained two genotypes, whereas the other (Lap-a/b) contained three genotypes, and both of them combined to form five different genotypes in total (Table 4).

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hk-a/b</td>
<td>11/11</td>
</tr>
<tr>
<td></td>
<td>11/11</td>
</tr>
<tr>
<td></td>
<td>11/22</td>
</tr>
<tr>
<td></td>
<td>11/22</td>
</tr>
<tr>
<td></td>
<td>11/22</td>
</tr>
<tr>
<td>Lap-a/b</td>
<td>11/33</td>
</tr>
<tr>
<td></td>
<td>33/33</td>
</tr>
<tr>
<td></td>
<td>11/33</td>
</tr>
<tr>
<td></td>
<td>23/33</td>
</tr>
<tr>
<td></td>
<td>33/33</td>
</tr>
<tr>
<td>Observation %</td>
<td>5</td>
</tr>
</tbody>
</table>

**Spore germination in darkness**

Spores of *L. thunbergianus* did not germinate in the dark without treatment, neither did they germinate on the media containing GA3 or 2% and 10% extracts of *L. thunbergianus* gametophyte cultures. Although the germination rates were not significantly different from the dark control, other treatments did induce some spores of *L. thunbergianus* to germinate in the dark. (Tables 5, 6). Antheridium promotion for all effective treatments was similar (Tables 5, 6). Most of the dark-growing gametophytes produced antheridia at very young stage when they possessed only one or two prothallial cells (Figs. 23-25) which were pale-white, although a few of the gametophytes remained asexual (Fig. 22). Many antheridia were mature when observed and released active sperms (Figs. 23, 24).

**Antheridium promotion in *Onoclea sensibilis***

Most of the gametophytes of *Onoclea sensibilis* generated antheridia 0.5 months after the spores were sown on media containing extract of *L. thunbergianus* or when spores were sown on blocks supporting mature gametophytes of *L. thunbergianus*, but all of them
Table 5. Average dark germination percentage and male percentage in *L. thunbergianus* under different conditions (experiment I) at one month after sowing spores.

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Germination %**</th>
<th>Male %**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Light control</td>
<td>85 (a)</td>
<td>0 (c)</td>
</tr>
<tr>
<td>2. Dark control</td>
<td>0 (b)</td>
<td>0 (c)</td>
</tr>
<tr>
<td>3. Lt 2% extract</td>
<td>0 (b)</td>
<td>0 (c)</td>
</tr>
<tr>
<td>4. Lt turned block</td>
<td>9 (b)</td>
<td>67 (b)</td>
</tr>
<tr>
<td>5. Pt 2% extract</td>
<td>2 (b)</td>
<td>60 (b)</td>
</tr>
<tr>
<td>6. Pt turned block</td>
<td>6 (b)</td>
<td>90 (a)</td>
</tr>
</tbody>
</table>

* Lt = *Lepisorus thunbergianus*, Pt = *P. aquilinum*.  
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95%c.l.). SEM = 1.52, and 10.21 for germination % and male % respectively, for conditions 3-6.

Table 6. Average dark germination percentage and male percentage in *L. thunbergianus* under different conditions (experiment II) at one month after sowing spores.

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Germination %**</th>
<th>Male %**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Light control</td>
<td>62 (a)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>2. Dark control</td>
<td>0 (b)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>3. Lt inverted block</td>
<td>3 (b)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>4. Lt cleared block</td>
<td>6 (b)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>5. Pt inverted block</td>
<td>6 (b)</td>
<td>97 (a)</td>
</tr>
<tr>
<td>6. Pt cleared block</td>
<td>6 (b)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>7. Lt 10% extract</td>
<td>0 (b)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>8. Pt 10% extract</td>
<td>2 (b)</td>
<td>89 (a)</td>
</tr>
<tr>
<td>9. GA3 5x10^-5 g/ml</td>
<td>0 (b)</td>
<td>0 (b)</td>
</tr>
</tbody>
</table>

* Lt = *Lepisorus thunbergianus*, Pt = *P. aquilinum*.  
** The same letter in parentheses in the same column indicates no significant difference Duncan's multiple test (95%c.l.). SEM = 0.96, and 4.56 for germination % and male % respectively, for conditions 3-8.

remained asexual under control conditions (Tables 7, 8). At one month and 1.5 months after sowing spores, a few gametophytes growing under the control condition produced antheridia but significantly fewer than those growing under treatment conditions (Tables 7, 8). The influence of the treatments was only slightly different at 0.5 months, and later became equa.
Figs. 24-29. Gametophyte response to experimental treatments. Figs. 24-27. *Lepisorus thunbergianus*. Figs. 24-25. Spore germination in darkness. Fig. 24. A dark-growing gametophyte on a cleared block of mature *L. thunbergianus* gametophytes. Fig. 25. A dark-growing gametophyte on a turned block of mature *L. thunbergianus* gametophytes. Figs. 26-27. Antheridium promotion by treatments. Fig. 26. A 2 month old gametophyte growing on an inverted block of mature gametophytes of *P. aquilinum*. Fig. 27. A 1.5 month old gametophyte growing on an inverted block of mature *L. thunbergianus* gametophytes. Figs. 28-29. *Onoclea sensibilis*. Fig. 28. A 0.5 month old *O. sensibilis* gametophyte growing on medium containing 2% extract of *L. thunbergianus* gametophyte cultures. Fig. 29. A 1 month old *O. sensibilis* gametophyte growing on an inverted block of mature *L. thunbergianus* gametophytes. Bar = 0.1 mm.
Table 7. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 months</td>
<td>1 month</td>
<td>1.5 months</td>
<td></td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (c)**</td>
<td>2 (b)</td>
<td>10 (b)</td>
<td></td>
</tr>
<tr>
<td>2. Lt. 2% extract</td>
<td>78 (b)</td>
<td>90 (a)</td>
<td>92 (a)</td>
<td></td>
</tr>
<tr>
<td>3. Lt. turned block</td>
<td>100 (a)</td>
<td>100 (a)</td>
<td>100 (a)</td>
<td></td>
</tr>
</tbody>
</table>

* Lt = *Lepisorus thunbergianus*.  
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 7.07, 5.24, and 4.83 for 0.5 months, 1 month, and 1.5 months respectively, for conditions 2 & 3.

Table 8. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 months</td>
<td>1 month</td>
<td>1.5 months</td>
<td></td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (d)**</td>
<td>6 (b)</td>
<td>17 (b)</td>
<td></td>
</tr>
<tr>
<td>2. Lt 10% extract</td>
<td>58 (c)</td>
<td>92 (a)</td>
<td>92 (a)</td>
<td></td>
</tr>
<tr>
<td>3. Lt cleared block</td>
<td>75 (b)</td>
<td>100 (a)</td>
<td>100 (a)</td>
<td></td>
</tr>
<tr>
<td>4. Lt inverted block</td>
<td>92 (a)</td>
<td>100 (a)</td>
<td>100 (a)</td>
<td></td>
</tr>
</tbody>
</table>

* Lt = *Lepisorus thunbergianus*.  
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 5.12, 4.00, and 4.00 for 0.5 months, 1 month, and 1.5 months respectively, for conditions 2-4.

(Tables 7, 8). Antheridia were produced from very young filaments or spathulate stage through heart-shaped prothalli. Usually only a few antheridia appeared on the young stages (Figs. 16, 28), but they were more abundant on the relatively larger gametophytes (Fig. 17, Fig. 29) and active sperms were released (Fig. 29).
Antheridium promotion in *Lepisorus thunbergianus*

GA3, and 2% and 10% extracts of either *L. thunbergianus* or *P. aquilinum* gametophyte cultures did not significantly promote antheridium formation (Tables 9, 10). The secretions of living gametophytes of *L. thunbergianus* significantly promoted antheridium formation at 1.5 months (Table 9), or 2.5 months (Table 10). Antheridium formation in *L. thunbergianus* was significantly promoted at 1.5 months by secretions of living gametophytes of *P. aquilinum* (Tables 9, 10). Under the effective treatment conditions, small spathulate gametophytes began to produce antheridia and remained at these sizes (Figs. 15, 26, 27). These antheridium producing gametophytes released abundant active sperms at 1.5 and 2 months. Sperms released from 2.5 months old treated gametophytes were relatively sluggish and these gametophytes gradually became brown and died while the control gametophytes still grew well.

**Discussion**

Gametophyte morphology of *Lepisorus* has been studied by Nayar (1961), Nayar and Raza (1970), Nayar and Kaur (1971), and Bhattacharyya and Sen (1992). Among these, Nayar and Raza (1970) reported that *L. thunbergianus* development was Drynaria-type and was elongated cordate as well as nearly strap-like. In this observation, based on the definition of Nayar and Kaur (1969; 1971), the development of *L. thunbergianus* is of Drynaria-type and concordant with previous studies. However, the mature gametophytes were broad cordate, and elongated strap-like to repeatedly branched. The branching behavior of these gametophytes likely tends to increase their living space occupied by gametophytes and prolong their life span. These extended clonal and perennial gametophytes continuously form gametangia on their new proliferations and thus enhance the possibility of interaction with other gametophytes establish previously or later.
Table 9. Average antheridium formation (%) by *L. thunbergianus* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>2 (c)**</td>
</tr>
<tr>
<td>2. Lt 2% extract</td>
<td>0 (c)</td>
</tr>
<tr>
<td>3. Pt 2% extract</td>
<td>2 (c)</td>
</tr>
<tr>
<td>4. Lt turned block</td>
<td>12 (b)</td>
</tr>
<tr>
<td>5. Pt turned block</td>
<td>92 (a)</td>
</tr>
</tbody>
</table>

* Lt = *Lepisorus thunbergianus*, Pt = *P. aquilinum.*
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95%c.l.). SEM = 4.47, 2.83, and 2.00 for 1.5 months, 2 months, and 2.5 months respectively, for conditions 2-5.

Table 10. Average antheridium formation (%) by *L. thunbergianus* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (c)**</td>
</tr>
<tr>
<td>2. GA3 5x10^-5 g/ml</td>
<td>0 (c)</td>
</tr>
<tr>
<td>3. Lt 10% extract</td>
<td>0 (c)</td>
</tr>
<tr>
<td>4. Pt 10% extract</td>
<td>0 (c)</td>
</tr>
<tr>
<td>5. Lt cleared block</td>
<td>10 (c)</td>
</tr>
<tr>
<td>6. Lt inverted block</td>
<td>13 (c)</td>
</tr>
<tr>
<td>7. Pt cleared block</td>
<td>54 (b)</td>
</tr>
<tr>
<td>8. Pt inverted block</td>
<td>92 (a)</td>
</tr>
</tbody>
</table>

* Lt = *Lepisorus thunbergianus*, Pt = *P. aquilinum.*
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95%c.l.). SEM = 9.58, 12.33, and 5.26 for 1.5 months, 2 months, and 2.5 months respectively, for conditions 2-8.
Intergametophytic mating has been recently thought to be the most common breeding system in homosporous ferns, and genetic load has been suggested to be the primary mechanism promoting outcrossing (Masuyama 1979, 1986; Haufler et al. 1990; Haufler and Welling 1994). The high genetic load in this culture seems to indicate that sporophytes of *L. thunbergianus* are mainly derived from intergametophytic mating.

Masuyama *et al.* (1987) studied the relationship of the mating system and ploidy of *L. thunbergianus* in Japan. They found that the gametophytes of diploid plants of this species produced few sporophytes by selfing and that most of their offspring were abnormal, whereas those of the tetraploid were almost totally successful (98-100%) and all of the sporophytes were normal. It seems very likely that the plants used in these culture studies were also diploid. Compared to the results of Masuyama *et al.* (1987), sporophyte production by selfing in this study was a little higher than that of their diploids but much lower than that of their tetraploid materials. Because of low sporophyte production in the paired-culture as well as in isolated-culture in this observation, we suspect that a very high number of recessive lethals are causing sporophyte depression in both intra- and intergametophytic selfing (since all gametophytes were from a single sporophyte in this observation).

Strong evidence for the mating system of diploid species in the wild can be derived from isozyme electrophoretic patterns. Electrophoretic patterns for *L. thunbergianus* revealed a high level of fixed interlocus heterozygosity, indicating that these samples from Hawaii are polyploid, probably allopolyploid. Because of this, accurate counts of heterozygous individuals and estimates of outcrossing was not possible, However, at least one outcrossed individual (Lap-a^{23/b^{33}}) was among the 40 samples.

Assuming allopolyploidy, the maximum number of genotypes per locus-pair indicates the minimum number of hybridization events involved in producing the species (Ranker *et al.* 1994). Thus three genotypes at Lap-a/b suggest that the population originated from at
least three hybridization events. On the other hand, the total number of multilocus genotypes estimates the actual number of hybridization events (Ranker et al. 1994), thus the total of five genotypes suggest that the independent hybridization events might have occurred as many as five times, assuming that sexual recombination or mutation plays only a minor role.

Antheridiogen has been considered to be a mechanism promoting outcrossing (Haufler and Welling 1994). Antheridium formation in Onoclea sensibilis gametophytes was promoted significantly by extracts of L. thunbergianus gametophyte cultures and when spores are sown near L. thunbergianus gametophytes (Tables 7, 8). In addition, antheridium production by L. thunbergianus gametophytes was promoted significantly when spores were sown near the mature gametophytes of L. thunbergianus. These results suggest that gametophyts of L. thunbergianus produce and respond to their own antheridiogen (ALE) and respond to antheridiogen of P. aquilinum (APT). ALE apparently is not similar to GA3 because the latter did not enhance antheridium formation. Furthermore, the response of L. thunbergianus gametophytes to antheridiogen of Pteridium aquilinum (APT) indicates that ALE may be more closely related to APT. The fact that 2% and 10% extracts of gametophyte cultures of L. thunbergianus and P. aquilinum did not efficiently facilitate the antheridium formation is possibly due to an insufficient antheridiogen concentration. The higher antheridium induction of living gametophytes of P. aquilinum than those of L. thunbergianus by secretions is possibly because gametophytes of L. thunbergianus are more sensitive to antheridiogen APT or because there is a higher concentration of antheridiogen in those treatments of P. aquilinum. The non-promotion of antheridium formation by extracts of L. thunbergianus cultures may also because that ALE is a short-lived antheridiogen or loses activity during the harvest process (Emigh and Farrar 1977). However, the fact that all treatments efficiently promoted antheridium formation in Onoclea sensibilis suggests that the antheridiogen ALE is a stable compound.
Another indirect evidence of antheridiogen of *L. thunbergianus* comes from the pattern of male expression in multi-spore cultures, where male gametophytes are smaller and antheridia are produced later than archegonia. This fits the model of the antheridiogen function proposed by Naf (1963) who suggested that the fast-growing females secrete antheridiogen which induces slow-growing gametophytes to produce antheridia.

Dopp (1950, cited from Naf 1963) found that the active preparation also retarded gametophyte growth except at very low concentration. Similarly, in this experiment, all the gametophytes growing in the effectively treated cultures were smaller than those in untreated cultures (data not shown). We here propose two possible explanations for the smaller gametophytes. One possibility is that the potential vegetative growth is diverted to antheridium production as demonstrated by Naf (1956). Although this can explain the stronger effect on small gametophytes growing near other mature gametophytes, it does not explain why asexual gametophytes growing in the treatment conditions are of similar sizes to male gametophytes. Another possibility is that inhibitor substances are secreted from the mature gametophytes as shown in some species of *Dryopteris* (Bell 1958). To test this, we sowed spores of *L. thunbergianus* on media treated as conditions 5 and 6 in the table 9, but using mature gametophytes of *Anemia phyllitidis* instead of *L. thunbergianus* as the antheridiogen source. *A. phyllitidis* is known to produce another type of antheridiogen (AAa) which induces antheridiogen production in *Anemia* but is inactive in families other than the Schizaeaceae (Naf et al. 1975). The size of *L. thunbergianus* in these cultures remained very small, similar in size to the precocious male gametophytes described above, but all remained asexual during the 3-month culture (data not shown). These results indicate that inhibitors may be universal and closely related among fern gametophytes, unlike antheridiogens which are more specific. It seems that *L. thunbergianus* can produce its own antheridiogen as well as inhibitor, and respond to both of them as well as to those produced by *P. aquilinum*. On
the other hand, for \textit{A. phyllitidis}, \textit{L. thunbergianus} responds to its inhibitor but does not responds to the antheridiogen \textit{AA_{An}}. An alternative hypothesis is that antheridiogen functions in both antheridium promotion and gametophyte growth inhibition, but the efficiency of these two functions is different depending on the species. The statement that "antheridiogens actually affect size, and size influences sex expression" (Korpelainen 1994) is questioned here, at least in this species.

In the Polypodiaceae, neither antheridiogen presence nor response to \textit{A_{Pl}} has been reported (Voeller 1971; Raghavan 1989), except for a very weak response in \textit{Aglaomorpha meyeniana} (Naf 1966; 1969). However, Welling and Haufler (1993) demonstrated antheridiogen response in \textit{Polypodium austral}. Haufler \textit{et al.} (1995) found that the frequency of outcrossing was consistent with random mating in the \textit{Polypodium vulgare} complex, a species (complex) thought to be unaffected by antheridiogen (Voeller 1964; Welling and Haufler 1993), and further pointed that this (assuming no antheridiogen system in \textit{Polypodium vulgare}) contradicts the viewpoint that "antheridiogen response and genetic load were coordinated in a predictable manner", stated by Schneller \textit{et al.} (1990). Presence of an antheridiogen response and high genetic load in \textit{L. thunbergianus} support the statement by Schneller \textit{et al.}, and suggests the need for further tests for presence of antheridiogens in \textit{Polypodium}.

In \textit{L. thunbergianus}, the existence of an antheridiogen response should increase the probability of intergametophytic mating by increasing the number of male plants in a population, thus maintaining genetic diversity on genetic load. It is possible that the plants used for culture studies were diploid, but this seems unlikely since all plants tested produced electrophoretic patterns consistent with tetraploidy. Assuming the spores tested were from tetraploid plants, these results indicate that polyploidization does not always lead to intragametophytic selfing and loss of genetic load.
Spores of *L. thunbergianus* were not induced to germinate under darkness by extracts of *L. thunbergianus* gametophyte cultures, but were induced by other treatments. Although the response was weak, it did indicate that there is a substance produced by gametophytes substituting for light requirement of spore germination. The lack of induction by extracts of *L. thunbergianus* cultures may be due to insufficient amounts of active chemical of *L. thunbergianus* in the extracts or because the chemical is short-lived or lost activity during the extraction process. On the other hand, the fact that extracts of *P. aquilinum* cultures promoted spores of *L. thunbergianus* to germinate in darkness suggested that this substance is stable, at least in *P. aquilinum*. In general, the functions of the substance for promoting antheridium formation and the substance for substituting for a light requirement for spore germination are parallel (Voeller 1971), but Welling and Haufler (1993) hypothesized that these two substances may function independently. In our experiments, all secretions of living gametophytes promoted both spore dark germination and antheridium production in *L. thunbergianus*, but extracts of *L. thunbergianus* gametophyte cultures did not. Thus the two functions are parallel in treatments of *L. thunbergianus*.

A soil spore bank has been demonstrated to exist for terrestrial species (Dyer and Lindsay 1992). For epiphytic species, fern spores are possibly deposited very deep in bryophyte mats where light intensity may be insufficient for spore germination. Antheridiogen (or germinin) may function in inducing germination of those "buried" spores. Through the antheridiogen influence, the resultant gametophytes could precociously produce antheridia and release active sperms which could interact with older gametophytes. This model has been proposed previously (Voeller 1971; Schneller *et al.* 1990; Haufler and Welling 1994) for terrestrial ferns, and may fit epiphytic species as well.

In summary, in *L. thunbergianus*, a tropical epiphytic fern, gametophytes are able to survive perennially through continuous branching and proliferations. Multiple bands and
fixed heterozygosity at most enzyme loci indicates polyploidy, but nevertheless, isolated gametophyte cultures demonstrated presence of a high genetic load. Antheridiogen (or germinin) can induce spores buried in humus or bryophyte mats to germinate and increase the number of male plants and thus the number of intergametophytic mating. The antheridiogen system, extended clonal proliferations, and the prolonged life span promote the interaction and outcrossing with other gametophytes which may develop from spores either dispersed later or previously buried in the epiphyte substrate.

Acknowledgments

We thank Dr. Paul N. Hinz for help with statistic analysis, Mr. William Norris for reviewing the manuscript, Mrs. Ming-Ren Huang for help with plant cultures, data recording, and manuscript preparation. This research was supported by the Department of Botany, Iowa State University, and by the National Science Council of Taiwan, Republic of China.

Literature cited


4. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF *MICROGRAMMA HETEROPHYLLA* (L.) Wherry

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**Introduction**

The morphology of fern gametophytes has been studied and applied to taxonomy and systematics for a long time. Beyond the standard heart shapes depicted in most textbooks, great variations have also been shown (Atkinson 1973; Nayar and Kaur 1971). These variations include the ability to grow indeterminately and branch so that perennial gametophyte clones of considerable size may be produced (Farrar 1990; Dassler 1995). The morphological diversity may relate to the species' mating system and reproductive biology.

Four types of mating system in homosporous pteridophytes have been defined by Klekowski (1979): (1) intragametophytic selfing, (2) intergametophytic selfing, (3) intergametophytic crossing, and (4) intergametophytic mating. Among these mating systems, intragametophytic selfing had been previously suggested as being predominant in homosporous ferns due to the close proximity of antheridia and archegonia on bisexual gametophytes (Klekowski and Baker 1966; Klekowski 1973, 1979) and indeed there are some homosporous ferns which have very high rates of intragametophytic selfing (Crisr and Farrar 1983; McCauley *et al.* 1985; Soltis and Soltis 1986a; Watano and Sahashi 1992). Polyploid species may also favor intragametophytic selfing due to duplicated alleles which mitigate the problem of recessive deleterious allele expression associated with selfing (Klekowski and Baker 1966; Lloyd 1974; Masuyama and Watano 1990). Recently, however, electrophoretic evidence has shown that intergametophytic mating is the most common breeding system in homosporous ferns (Haufler and Soltis 1984; Holsinger 1987; Soltis and Soltis 1986b; Soltis and Soltis 1990; Ranker 1992).
Several reproductive behaviors can reduce the probability of intragametophytic selfing. Asynchronous maturation of male and female gametangia is one way to avoid intragametophytic selfing in bisexual gametophytes (Klekowski 1968, 1969; Masuyama 1974a, b). Possession of an antheridiogen system is another way to facilitate production of unisexual gametophytes, and thus promote intergametophytic mating (Naf et al. 1975; Voeller 1971; Hamilton and Lloyd 1991). Antheridiogen (or germinin, proposed by Voeller 1971) also overcomes the inhibition of spore germination in darkness (Voeller 1971; Schneller et al. 1990). However, except for Polypodium australe (Welling and Haufler 1993), and Agluomorpha meyeniana (Naf 1966; 1969), Polypodiaceae s.s. has been thought neither to produce its own antheridiogen nor to respond to the antheridiogen of Pteridium aquilinum (L.) Kuhn, the most common antheridiogen known (Voeller 1971; Raghavan 1989). Genetic load is also an obstacle to successful intragametophytic selfing, and the degree of genetic load has been used to measure the sporophyte heterozygosity and probable breeding system (Lloyd 1974; Cousens 1988). However, most of the information known about the reproductive biology of ferns is from research on terrestrial ferns which encounter very different environmental events from epiphytic ferns.

Microgramma heterophylla (L.) Wherry, a member of the Polypodiaceae, is a rare epiphytic species distributed in Florida and the West Indies (Lellinger 1985; Nauman 1993). Neither the gametophyte morphology nor the reproductive biology has been previously studied. Due to the epiphytic habitat of this species and the nature of its bark and bryophyte substrate, dispersed spores are likely to be more widely separated, and the interaction of the subsequent gametophytes more hindered than in terrestrial species (Dassler 1995). Thus gametophytes of epiphytic species might be more likely to produce their sporophytes through intragametophytic selfing. This paper investigates this issue in Microgramma heterophylla, addressing the following questions: (1) how are gametangia expressed in this species, (2)
how much genetic load exists, (3) do gametophytes produce and/or respond to antheridiogen, (4) through which mating system are sporophytes produced, (5) how does gametophyte morphology relate to the reproductive system.

Materials and methods

Spores of *Microgramma heterophylla* were collected from the greenhouse of the Department of Botany, Iowa State University. This plant was collected by D.R.F. in Dade county, Florida, in September, 1968. Since antheridiogen of *Pteridium aquilinum* (L.) Kuhn (Apt) has been well studied (Naf et al. 1975), and gametophytes of *Onoclea sensibilis* L. have been used to "safely identify antheridium-inducing activity" due to their complete lack of spontaneous antheridium production in young stages (Naf 1956), these two species were also used in this study to assay the activity of antheridiogen of *M. heterophylla*. Spores of *Onoclea sensibilis* were collected from Marshall County, Iowa, in September, 1994 (Chiou s.n.), and those of *Pteridium aquilinum* were in part collected in June, 1991 from Iowa City, Iowa (Farrar 91-6-26-27), and in part were kindly sent by Dr. David Wagner, collected from Eugene, Oregon, in August 3, 1994. Voucher specimens of *M. heterophylla* were deposited in the Ada Hayden Herbarium (ISC) of the Department of Botany in Iowa State University.

Spores obtained from fertile fronds (Chiou and Farrar 1994) were stored in the refrigerator at about 5°C.

Spores were sown on 1% agar solidified media which contained Bold's macronutrients (Bold 1957) and Nitsch's micronutrients (Nitsch 1951), and a trace of ferric chloride (Peck 1985). Cultures were maintained under continuous, white fluorescent illumination of 2000-3000 lux. Temperature was maintained between 20-24°C.

Gametophyte morphology was observed under dissecting and compound microscopes. Pictures were either taken by auto-camera or hand-drawn using a drawing tube.
Sizes of gametophytes were measured as the width of the widest part. Gametophytes were observed every half month.

To harvest antheridiogen-enriched solution, the following procedure was used. One mg of spores of *M. heterophylla* and *P. aquilinum* were separately sterilized with 10% Clorox for 5 minutes and then rinsed with sterilized water. The sterilized spores were then suspended in 2 ml of sterilized water. Two drops of the spore suspension were placed into each of several petri dishes of 8.5 cm diameter containing about 50 ml of agar media. The cultures of *M. heterophylla* and *P. aquilinum* gametophytes were frozen at 8 months and 2 months old respectively, then thawed and the liquid extract collected. 2% and 10% dilutions of the extract were made by adding 20 drops (ca. 1 ml) of the extract into large petri dishes containing 50 ml of agar medium and the small petri dishes containing 10 ml of agar medium respectively. In addition to these extracts, GA3 (5x10^-5 g/ml) was also used to test antheridiogen response.

Considering the possibility that some antheridiogens might be short-lived (Emigh and Farrar 1977), that the time of harvesting extract might be not proper, or that the harvesting method may destroy antheridiogen activity, three additional experiments were conducted in which agar with mature growing gametophytes was used directly for the assay. In the first experiment, the agar with growing mature gametophytes was cut into 1 x 2 cm blocks. Then each agar block was turned on its side (turned-block), so the gametophytes stood on one side of the agar block. Ten spores were sown on the top of each block and ten such blocks were put into each of two petri dishes. In the second experiment, agar supporting mature gametophytes was cut into four parts. Each part was placed upside down in another petri dish, and spores were sown on the upside-down agar (inverted-block). In the third experiment, some gametophytes were removed from cultures to make a 1 cm wide lane (cleared-block), and spores were sown on these cleared areas (between mature gametophytes). All
gametophyte cultures assayed for presence of antheridiogen were 8-month- and 2-month-old of *M. heterophylla* and *P. aquilinum* respectively.

The preparation of both extracts and blocks with growing gametophytes were used in tests of germination in darkness and promotion of antheridium formation in the light. A complete randomized design was used. The percentage of plants forming antheridia was counted as the number of male plus bisexual individuals. Tests were repeated twice in the first experiment (Tables 5, 7) and four times in the second and third experiments (Tables 6, 8). At each observation time, 25 gametophytes were removed from each dish in the first experiment and 12 plants were removed from each dish in the second and third experiments.

Capability of spore germination in darkness and the promotion of spore germination in the dark by antheridiogen were tested by wrapping two layers of aluminum foil around spore-sown petri dishes. These wrapped dishes were placed in a dark cabinet, and unwrapped and examined one month later. Tests were repeated two (table 3) and three (table 4) times. Germination percentage was determined by counting 100 spores. The proportion of male plants was measured as the number of male gametophytes divided by the total number of dark-germinated gametophytes. A complete randomized design was used.

Genetic load was calculated by comparing isolated-spore and isolated-gametophyte cultures with paired-spore and paired-gametophyte cultures. These cultures were grown on "jelly-mold" plates, each with 20 cells. Each cell contained about 6 ml of agar medium. In each plate, a single spore was transferred onto each of 5 cells, and an one-month-old gametophyte which was still asexual was transferred onto each of another 5 cells. Another 10 cells were used as paired-spore and paired-gametophyte controls. Five such plates were separated by transparencies and stacked into transparent plastic vegetable crispers for a total of 50 replicates of each culture type. The light intensity of these cultures were maintained between 1500 lux (the bottom layer) to 3500 lux (the top layer). Plants were watered every
two weeks after gametophytes were 4 month old. Sporophytes were determined to have been produced sexually by examination with a compound microscope. Genetic load was measured by counting the percentage of bisexual gametophytes failing to produce sporophytes. The five layers of plates were designated as blocks, and spore-culture vs. gametophyte-culture were designated as a split plot.

Results

Morphology

In multispore cultures, spores began to germinate one week after sowing spores. Viability of spores remained at 58% through 1 year of storage in the refrigerator at 5°C, but the germination rate declined to 12% and the beginning of germination was delayed to about two weeks in spores that were stored more than 1.5 years. After the basal cell, the rhizoid was usually the first cell produced on germination (Fig. 1). The basal cell underwent a second division (Fig. 2), resulting in a protonemal cell which subsequently produced a filament by serial divisions (Fig. 3). The lengths of filaments varied from four to eight cells. Sometimes, a short branch appeared but did not develop further (Fig. 4). In addition to the basal cell, rhizoids also emerged from other cells of the filament below the top cell.

About one week after germination, a longitudinal division usually occurred at either the apical cell or the cells below it (Figs. 5, 6), and a subsequent spathulate prothallus resulted through apical growth (Figs. 8, 9) or intercalary growth (Fig. 7). Sometimes all the filament cells except the basal cell divided longitudinally (Fig. 8). Eventually an obconical meristematic cell was built at the anterior margin by two oblique divisions (Fig. 10).

Later, a pluricellular meristem replaced the single meristematic cell, and finally a nearly symmetrical cordate gametophyte formed (Fig. 11). In some individuals, a lateral or sublateral meristem differentiated (Fig. 12) if the top cell developed a hair (Fig. 7). Most gametophytes attained a width of about 3-4 mm, then became elongated.
Figs. 1-20. Gametophyte development in *Microgramma heterophylla* in culture populations. Figs. 1-12. Germination and early development. Figs. 13, 14. Superficial hairs. Fig. 15. Unicellular hair on a marginal cell. Figs. 16-18. Multicellular hairs. Fig. 19. Branched hermaphrodite gametophyte. Fig. 20. A small protrusion with a hair on the top. An = antheridium. Ar = archegonium. Bar = 0.1 mm, unless otherwise indicated.
In addition to the margin, rhizoids also occurred on both dorsal and ventral sides of the midrib as well as on the wings, but those on the latter were fewer than those on the midrib or on the margin.

Hairs began to appear on spathulate prothalli. They grew on the margins and occasionally on both dorsal and ventral sides of wings and midribs. The former were unicellular, papillate, and secretory (Fig. 15), whereas the latter were multicellular and club-shaped (Figs. 13, 14). Marginal hairs appeared earlier than superficial hairs (Figs. 11, 12). Usually a gametophyte cell developed only one hair but sometimes two hairs could be found on one cell (Fig. 7). On older gametophytes, longer, multicellular, and club-shaped hairs also emerged on the margin (Figs. 16-18).

At about 1.5 months, antheridia appeared, usually on the posterior part (Fig. 23), sometimes on the margin or near the apical meristem if a midrib had not formed (Fig. 21). Archegonia, also appeared at about 1.5 months, were distributed on the midrib behind the meristem (Figs. 19, 22-24). Both antheridia and archegonia were present on both ventral and dorsal sides.

As gametophytes grew older, sections of the margin became active and produced additional meristems (Fig. 19). From these meristems, branches formed and produced additional meristems. Branches arising from one or a few cells also occurred (Figs. 20, 34). A mat of overlapping branches thus formed (Fig. 33). Branches were usually relatively narrow and elongated (Fig. 33). Gametangia continuously formed on the new branches. Gametophytes continued growing over two years and several sporophytes were derived from many individual gametophytes, which still remained growing after sporophyte production.

Sex sequence

In multispore cultures, gametangia did not appear until two months after sowing spores. Sexual frequency varied with culture ages (Table 1). Archegonia developed earliest
Figs. 21-32. Gametangia expression. Figs. 21-24. *Microgramma heterophylla* in population cultures. Fig. 21. A male gametophyte. Fig. 22. A female gametophyte. Fig. 23. A hermaphroditic gametophyte. Fig. 24. Part of a branch with antheridia and archegonia. Figs. 25-32. Gametophyte response to experimental treatments. Figs. 25-30. *M. heterophylla*. Fig. 25. Dark-growing gametophytes on an inverted-block of *M. heterophylla* gametophytes. Fig. 26. Dark-growing gametophytes on an inverted-block of *Pteridium aquilinum* gametophytes. Fig. 27. 1.5 month old gametophyte growing on a cleared-block of *P. aquilinum* gametophytes. Fig. 28. 2 month old gametophyte growing on agar media containing 2% extraction of *P. aquilinum* gametophyte. Fig. 29. 1.5 month old gametophyte growing on an inverted-block of *M. heterophylla* gametophytes. Fig. 30. 1.5 month old gametophyte growing on a cleared-block of *M. heterophylla* gametophytes. Figs.31-32. *Onoclea sensibilis* gametophytes. Fig. 31. 0.5 month old gametophyte of *O. sensibilis* growing on media containing 2% extract of mature *M. heterophylla* gametophyte culture media. Fig. 32. 1 month old gametophyte of *O. sensibilis* growing on media containing 10% extract of mature *M. heterophylla* gametophyte culture media. An= antheridium. Ar = archegonium. Bar = 0.1 mm, unless otherwise indicated.
Figs. 33-36. Gametophyte development and experimental response of *Microgramma heterophylla*. Fig. 33. A clone of *M. heterophylla*. Fig. 34. New branches developing from an older branch. Fig. 35. A dark-growing gametophyte on an agar medium containing 2% extract of *P. aquilinum* gametophyte culture media. Fig. 36. A dark-growing gametophyte on a cleared-block of *M. heterophylla* gametophytes. Bar = 1 mm for Fig. 33; bar = 0.1 mm for Figs. 34-36.
Table 1. The sexual frequency (%) and gametophyte size of *M. heterophylla* at different ages in the multi-spore cultures.

<table>
<thead>
<tr>
<th>Width (mm)</th>
<th>2 months</th>
<th>2.5 months</th>
<th>3 months</th>
<th>4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A M F H*</td>
<td>A M F H</td>
<td>A M F H</td>
<td>A M F H</td>
</tr>
<tr>
<td>0.5</td>
<td>13 - - -</td>
<td>4 - - -</td>
<td>2 - -</td>
<td>4 - -</td>
</tr>
<tr>
<td>1</td>
<td>54 - - -</td>
<td>7 4 - -</td>
<td>4 6 -</td>
<td>4 6 -</td>
</tr>
<tr>
<td>2</td>
<td>19 2 4</td>
<td>4 - 8 -</td>
<td>- 2 2</td>
<td>- 2 4</td>
</tr>
<tr>
<td>3</td>
<td>- - 9</td>
<td>- - 42 11</td>
<td>- - 19</td>
<td>- - 10</td>
</tr>
<tr>
<td>&gt;=4</td>
<td>- - -</td>
<td>- - 17 4</td>
<td>- - 48</td>
<td>- - 46</td>
</tr>
<tr>
<td>Total</td>
<td>85 2 13</td>
<td>15 4 67 15</td>
<td>6 10 69 15</td>
<td>4 10 60 21</td>
</tr>
</tbody>
</table>

* A = asexual, M = male, F = female, H = hermaphrodite.

and female plants remained the most common during the culture period of 4 months. Males appeared a little later than females but earlier than the hermaphrodites, and remained relatively less frequency during the 4-month culture (Table 1). These male gametophytes were relatively small and seemed to be developing from slow growing individuals, perhaps late-germinated spores. Hermaphrodites developed last and gradually became the principal type after 4 months (data not shown). Due to their relatively large size, these hermaphrodites were most likely derived from female gametophytes (Table 1; Fig. 19), but some relatively smaller hermaphrodites (Fig. 23) might have been derived from male gametophytes.

Among isolated-cultures, sex expression percentages were not significantly different either among different plate layers or between the spore- and gametophyt-cultures (Table 2).

**Spore germination in darkness**

Spores of *M. heterophylla* did not germinate in the dark, or in darkness when GA3 was added, on media with extract of *M. heterophylla* gametophyte cultures, or on the turned-block growing with *M. heterophylla* gametophytes. On the other hand, spores of *M. heterophylla* germinated when the culture extracts of *P. aquilinum* were added to the medium (Table 3, condition 5; Table 4, condition 8), when spores were sown on side-turned blocks growing mature *P. aquilinum* gametophytes (Table 3, condition 6), or when spores were
Table 2. The sex expression (%) of *M. heterophylla* in isolated-spore (spore) and isolated-gametophyte (gametophyte) cultures at 8 months after sowing spores.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore</td>
<td>14 (a)</td>
<td>0</td>
<td>86 (a)</td>
</tr>
<tr>
<td>Gametophyte</td>
<td>6 (a)</td>
<td>0</td>
<td>94 (a)</td>
</tr>
</tbody>
</table>

[^1^] The same letter in parentheses means no significant difference in Duncan's multiple test (95% c.l.). SEM = 6.16 & 19.56 for ^1^ & ^2^ respectively.

sown on inverted or cleared-blocks growing mature gametophytes of *M. heterophylla* or *P. aquilinum* (Table 4, conditions 3-6). In general, the germination rates in darkness were relatively higher in treatments with *P. aquilinum*, either extract or growing gametophytes.

Antheridium promotion in all effective treatments were not significantly different (Tables 3, 4). Most dark-growing gametophytes produced antheridia at very young stages when they possessed only one or two prothallial cells (Figs. 25, 26, 35, 36) which were pale-white and sometimes relatively elongated (Fig. 35), although a few of these gametophytes remained asexual. Many of the antheridia were mature and released active sperms (Figs. 35, 36).

**Antheridium promotion in *Onoclea sensibilis***

Most of the gametophytes of *Onoclea sensibilis* generated antheridia by 0.5 months when spores were sown on media containing the extract of *M. heterophylla* gametophyte cultures or spores were sown near the mature gametophytes of *M. heterophylla*, whereas all of them remained asexual under control conditions (Tables 5, 6). At one month and 1.5 months after spores were sown, a few of the gametophytes growing under control conditions produced antheridia but significantly less than those growing under treatment conditions (Tables 5, 6). The influence of the treatments were only slightly different at 0.5 month, and later became non-significantly different (Tables 5, 6). Antheridia were produced from very
Table 3. Average dark germination percentage and male percentage of *M. heterophylla* under different conditions (experiment I) at one month after sowing spores.

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Germination %**</th>
<th>Male %**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Light control</td>
<td>58 (a)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>2. Dark control</td>
<td>0 (c)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>3. Mh 2% extract</td>
<td>0 (c)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>4. Mh turned-block</td>
<td>0 (c)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>5. Pt 2% extract</td>
<td>7 (c)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>6. Pt turned-block</td>
<td>31 (b)</td>
<td>100 (a)</td>
</tr>
</tbody>
</table>

*Mh = M. heterophylla, Pt = P. aquilinum.*

**The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 2.41, 0.00 for germination % and male % respectively, for conditions 3-6 only.

Table 4. Average dark germination percentage and male percentage of *M. heterophylla* under different conditions (experiment II) at one month after sowing spores.

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Germination %**</th>
<th>Male %**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Light control</td>
<td>12 (b)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>2. Dark control</td>
<td>0 (d)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>3. Mh inverted-block</td>
<td>8 (bc)</td>
<td>92 (a)</td>
</tr>
<tr>
<td>4. Mh cleared-block</td>
<td>6 (c)</td>
<td>93 (a)</td>
</tr>
<tr>
<td>5. Pt inverted-block</td>
<td>9 (bc)</td>
<td>93 (a)</td>
</tr>
<tr>
<td>6. Pt cleared-block</td>
<td>16 (a)</td>
<td>98 (a)</td>
</tr>
<tr>
<td>7. Mh 10% extract</td>
<td>0 (d)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>8. Pt 10% extract</td>
<td>10 (bc)</td>
<td>92 (a)</td>
</tr>
<tr>
<td>9. GA3 5x10^-5 g/ml</td>
<td>0 (d)</td>
<td>0 (b)</td>
</tr>
</tbody>
</table>

*Mh = M. heterophylla, Pt = P. aquilinum.*

**The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 1.52 and 5.37 for germination % and male % respectively, for conditions 3-8 only.
Table 5. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (b)**</td>
</tr>
<tr>
<td>2. Mh. 2% extract</td>
<td>96 (a)</td>
</tr>
<tr>
<td>3. Mh. turned-block</td>
<td>88 (a)</td>
</tr>
</tbody>
</table>

* Mh = *M. heterophylla*.  
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 2.83, 2.83, and 1.41 for 0.5 months, 1 month, and 1.5 months respectively, for conditions 2-3 only.

Table 6. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (c)**</td>
</tr>
<tr>
<td>2. Mh 10% extract</td>
<td>73 (b)</td>
</tr>
<tr>
<td>3. Mh cleared-block</td>
<td>88 (a)</td>
</tr>
<tr>
<td>4. Mh inverted-block</td>
<td>75 (b)</td>
</tr>
</tbody>
</table>

* Mh = *M. heterophylla*.  
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 4.69, 1.30, and 1.30 for 0.5 months, 1 month, and 1.5 months respectively, for conditions 2-4 only.

Young filaments or on spatulate through heart-shaped stages of prothallus development. Usually only few antheridia were produced at the young stages (Figs. 31, 39), but relatively more were seen later (Figs. 32, 40) and active sperms were released.

**Antheridium promotion in *Microgramma heterophylla***

Gametophytes of *M. heterophylla* produced a few antheridia both under control conditions and on the media to which GA3 was added 1.5 months, but the percentages of
Figs. 37-40. Experimental response. Figs. 37-38. *Microgramma heterophylla*. Fig. 37. A gametophyte growing on a cleared-block of *M. heterophylla* gametophytes. Fig. 38. A gametophyte growing on a turned-block of *P. aquilinum* gametophytes. Figs. 39-40. *Onoclea sensibilis* gametophytes. Fig. 39. A gametophyte growing on an agar medium containing 2% extract of *M. heterophylla* gametophyte culture media. Fig. 40. A gametophyte growing on a turned-block of *M. heterophylla* gametophytes. Bar = 0.1 mm.
male gametophytes were significantly lower than those of other treatments when observed at 1.5, 2, and 2.5 months (Tables 7, 8). The 2% extracts of either *M. heterophylla* or *P. aquilinum* gametophyte cultures promoted antheridium development less than the secretions of living gametophytes (Table 7). The 10% extracts of the two species gametophyte cultures also enhanced antheridium onset less than secretions of living gametophytes at 1.5 months, but these treatments were equally effective thereafter (Table 8). Under the effective treatment conditions, gametophytes began to produce antheridia at the filaments (Figs. 29, 30) through spathulate stage (Figs. 27, 37, 38) and remained at relatively small sizes, especially in block cultures. Gametophytes induced to produce antheridia precociously released abundant active sperms (Figs. 37, 38).

**Genetic load**

As shown in Table 9, gametophytes totally failed to produce sporophytes in isolated-spore cultures and nearly failed to do so in isolated-gametophyte cultures by 8 months after sowing spores. Thus the genetic load was very high, up to 99 in average, and there was no significant difference between monospore cultures and monogametophyte cultures. On the other hand, the paired-cultures were relatively successful in producing fertilized sporophytes (28% in average). Sporophyte production rates were not significantly different among different plate layers (data not shown).

**Discussion**

Gametophyte morphology of *M. heterophylla* has not been previously studied, but the prothallic development of Polypodiaceae has been previously issued as Drynaria-type and Kaulinia-type for cordate-shaped and ribbon-like gametophyte respectively (Nayar and Kaur 1971). In this observation, based on Nayar and Kaur (1969; 1971), the development of most *M. heterophylla* gametophytes are of the Drynaria-type. Some are of the Aspidium-type where the terminal cell produces a hair and the meristem then develops laterally.
Table 7. Average antheridium formation (%) by *M. heterophylla* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>1.5 months</th>
<th>2 months</th>
<th>2.5 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>2 (c)**</td>
<td>22 (c)</td>
<td>32 (c)</td>
</tr>
<tr>
<td>2. Mh 2% extract</td>
<td>48 (b)</td>
<td>58 (b)</td>
<td>68 (b)</td>
</tr>
<tr>
<td>3. Pt 2% extract</td>
<td>49 (b)</td>
<td>60 (b)</td>
<td>56 (b)</td>
</tr>
<tr>
<td>4. Mh turned-block</td>
<td>100 (a)</td>
<td>100 (a)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>5. Pt turned-block</td>
<td>100 (a)</td>
<td>100 (a)</td>
<td>100 (a)</td>
</tr>
</tbody>
</table>

* Mh = *M. heterophylla*, Pt = *P. aquilinum*.
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 4.72, 3.61, and 2.83 for 1.5 months, 2 months, and 2.5 months respectively, for conditions 2-5 only.

Table 8. Average antheridium formation (%) by *M. heterophylla* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>1.5 months</th>
<th>2 months</th>
<th>2.5 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>2 (d)**</td>
<td>2 (b)</td>
<td>19 (b)</td>
</tr>
<tr>
<td>2. GA3 5x10^-5 g/ml</td>
<td>8 (d)</td>
<td>11 (b)</td>
<td>24 (b)</td>
</tr>
<tr>
<td>3. Mh 10% extract</td>
<td>88 (c)</td>
<td>100 (a)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>4. Pt 10% extract</td>
<td>90 (bc)</td>
<td>100 (a)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>5. Mh cleared-block</td>
<td>92 (abc)</td>
<td>100 (a)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>6. Mh inverted-block</td>
<td>96 (ab)</td>
<td>100 (a)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>7. Pt cleared-block</td>
<td>100 (a)</td>
<td>100 (a)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>8. Pt inverted-block</td>
<td>100 (a)</td>
<td>100 (a)</td>
<td>100 (a)</td>
</tr>
</tbody>
</table>

* Mh = *M. heterophylla*, Pt = *P. aquilinum*.
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 1.69, 0.00, and 0.00 for 1.5 months, 2 months, and 2.5 months respectively, for conditions 3-8 only.
Table 9. The percentage of sporophyte production and gene load (by surviving bisexual gametophytes) by 8-month-old *M. heterophylla* in spore-culture (spore) and gametophyte-culture (gametophyte).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% sporophyte</th>
<th>load</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spore</td>
<td>gametophyte</td>
</tr>
<tr>
<td>isolated</td>
<td>0(b)¹</td>
<td>32(a)²</td>
</tr>
<tr>
<td>paired</td>
<td>2(b)¹</td>
<td>24(a)¹</td>
</tr>
<tr>
<td></td>
<td>1(b)²</td>
<td>28(a)²</td>
</tr>
</tbody>
</table>

¹, ², ³ The same letter in parentheses means no significant difference in Duncan's multiple test (95% c.l.). SEM = 3.84, 2.59, and 1.41 for ¹, ², and ³ respectively.

The mature gametophytes of Polypodiaceae have been described as cordate, strap-like and ribbon-like (Nayar and Kaur 1971). For *M. heterophylla*, gametophytes are cordate at young and early mature stages, but older gametophytes become highly branched and do not fit into Nayar and Kaurs' (1971) classification. The branching behavior of these gametophytes likely tends to increase the space occupied by the gametophytes and to prolong their life span. These extended clonal and perennial gametophytes continuously form gametangia on their new proliferations, and thus enhance the possibility of interaction with other gametophytes established previously or later.

Intergametophytic mating has been recently thought to be the most common breeding system in homosporous ferns (Haufler and Soltis 1984; Soltis and Soltis 1990), and genetic load has been suggested to be the primary mechanism promoting outcrossing (Masuyama 1979, 1986; Haufler et al. 1990; Haufler and Welling 1994). The high genetic load in this study suggests that sporophytes of *M. heterophylla* are from a highly outcrossing population. Lloyd (1974) concluded that high levels of genetic load and intergametophytic mating characterize the species of the mature rain forest. Nauman (1993) described *M. heterophylla* as growing in tropical hammocks. Thus the habitat and the breeding system of *M. heterophylla* fits Lloyd's hypothesis. On the other hand, formation of a few sporophytes by
isolated gametophytes suggests that occasional intragametophytic selfing is still possible. This could give isolated gametophytes, derived from isolated spores, some chance to establish new populations.

Antheridiogen has been considered to be a mechanism promoting outcrossing (Haufler and Welling 1994). The results from tests on both *Onoclea sensibilis* and *M. heterophylla*, indicates that antheridium formation is promoted significantly when the extract from *M. heterophylla* gametophyte cultures was added to the media and when spores were sown near mature *M. heterophylla* gametophytes (Tables 5-8). We conclude that antheridiogen (*A*<sub>mh</sub>) exists in this species although it has not been purified and identified. *A*<sub>mh</sub> apparently is less similar to GAS (because the latter did not enhance antheridium formation) than it is to antheridiogen of *Pteridium aquilinum* (*A*<sub>pt</sub>), to which gametophytes of *M. heterophylla* responded with precocious antheridium formation.

Another indirect evidence of an antheridiogen system in *M. heterophylla* comes from the pattern of male expression in multi-spore cultures, where male gametophytes are smaller and antheridia are produced later whereas female gametophytes are larger and archegonia develop earlier. This fits the model of the antheridiogen function proposed by Naf (1963) who suggested that fast-growing females secrete antheridiogen which induces slow-growing gametophytes to produce antheridia.

Dopp (1950, cited from Naf 1963) found that antheridiogen extracts also retarded gametophyte growth except at very low concentration. In this experiment, similarly, all gametophytes growing in the effectively treated cultures were smaller than those in the other cultures (data not shown). In treated cultures, gametophytes derived from spores sown near other mature gametophytes (higher concentration of active substance) were smaller than those growing on medium containing extract (lower concentration of active substance). We here propose two possibilities to explain the smaller gametophytes. One is that the potential
vegetative growth is diverted to antheridium production as demonstrated by Naf (1956).
Although this can explain the stronger effect on smaller male gametophytes, it does not
explain why asexual gametophytes are of similar sizes to male gametophytes. Another
possibility is that an inhibitor substance is secreted from the mature gametophytes as shown
in some species of Dryopteris (Bell 1958). To test this, we sowed spores of M. heterophylla
on media treated as in conditions 5 and 6 in the table 8, but using mature gametophytes of
Anemia phyllitis instead of M. heterophylla. A. phyllitidis is known to produce another type
of antheridiogen (A\textsubscript{An}) which induces antheridium production in Anemia but is inactive in
families other than the Schizaeaceae (Naf et al. 1975). The size of M. heterophylla in these
cultures remained very small, similar in size to the precocious male gametophytes described
above, but all remain asexual during the 3-month culture (data not shown). These results
indicate that inhibitors may be universal and more closely related than antheridiogens among
fern gametophytes. It seems that M. heterophylla can produce its own antheridiogen as well
as inhibitor, and responds to both of them as well as to those produced by Pteridum aquilinum. On the other hand, for A. phyllitidis, M. heterophylla responds to its inhibitor but
does not respond to the antheridiogen A\textsubscript{An}. An alternative hypothesis is that antheridiogen
functions in both antheridium promotion and gametophyte growth inhibition, but the
efficiency of these two functions is different depending on the species. The statement that
"antheridiogens actually affect size, and size influences sex expression" (Korpelainen 1994),
is questioned here, at least in this species.

Based on laboratory results, Schneller et al. (1990) pointed out a correlation between
antheridiogen response and genetic load. For M. heterophylla, the high genetic load and the
existence of antheridiogen support this correlation.

In the polypodiaceae, neither antheridiogen presence nor response to A\textsubscript{Pt} has been
thought to exist (Voeller 1971; Raghavan 1989), except for a very week response in
Aglamorpha meyeniana (Naf 1966; 1969). Recently, Welling and Haufler (1993) also demonstrated antheridiogen response in Polypodium australe. *M. heterophylla* must be added to the list of species of Polypodiaceae possessing an antheridiogen system.

A substance secreted from mature gametophytes of *M. heterophylla* promotes spore germination in darkness, substituting for the requirement of light for spore germination (Tables 3, 4). We do not know if this substance is the antheridiogen *Amh* or the so-called germinin (Voeller 1971). In general, functions of the substance on promoting antheridium formation and the substance substituting for a light requirement for spore germination are usually parallel (Voeller 1971). However, Welling and Haufler (1993) hypothesized that these two substances might function independently.

In our experiments, media containing 2% or 10% culture extracts of *M. heterophylla* gametophytes failed to induce spores to germinate under darkness (Tables 3, 4) but did promote antheridium formation (Tables 7, 8). Several explanations are possible. First, antheridiogen and germinin may be different substances, and germinin may be short-lived, or lose activity in the process of extraction, or is required high concentration for spore germination in darkness. Thus the extract, which might contain no active germinin, and the turned-blocks, which perhaps contained insufficient amounts of germinin due to relatively few *M. heterophylla* growing gametophytes, did not induce spore germination in darkness, whereas inverted-blocks and cleared-blocks which contained more mature gametophytes did promote dark germination.

Antheridiogen and germinin may be the same substance, but the threshold concentration for inducing antheridium formation is less than that for substituting for the light requirement of spore germination. The fact that treatments of less amounts of antheridiogen, *i.e.*, culture extracts and turned blocks of *M. heterophylla*, did not induce spore germination in darkness but promote antheridium formation sustains this hypothesis.
Another evidence supporting this is the finding that the same treatment induced only a very low percentage of spore germination in darkness but promoted very high percentage of antheridium production (Table 4, conditions 3, 4 and Table 5, conditions 3, 4). However, darkness may also cancel a light-dependent antheridium block and allow antheridia to form spontaneously (Naf et al. 1974). Without more evidence, we cannot conclude whether antheridiogen and germinin are the same or different.

A soil spore bank has been demonstrated to exist for terrestrial species (Dyer and Lindsay 1992). For epiphytic species, fern spores are possibly deposited very deep in bark or bryophyte mats where light intensity may be insufficient for spore germination. Antheridiogen (or germinin), may function in inducing germination of these "buried" spores. Through the antheridiogen influence, the resultant precocious gametophytes could produce antheridia and release active sperms to interact with older gametophytes. This model has been proposed previously (Voeller 1971; Schneller et al. 1990; Haufler and Welling 1994) for the terrestrial ferns, and may fit epiphytic species as well.

In summary, *M. heterophylla*, a tropical epiphytic fern, produces gametophytes which are able to survive perennially through continuous branching and vegetative proliferation. Sporophytes are produced primarily from outcrossing due to a high level of genetic load. A clonal-forming habit, prolonged life span, and an antheridiogen system (possibly plus germinin), provides a mechanism to promote interaction and outcrossing with other gametophytes which may develop from spores either dispersed later or previously buried in the epiphytic substrate.

**acknowledgments**

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**Literature cited**


5. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF *PHLEBODIUM AUREUM* (L.) J. SMITH

This paper is prepared for publication in American Fern Journal

Wen-Liang Chiou¹, Donald R. Farrar¹, and Tom A. Ranker²

**Introduction**

The morphology of fern gametophytes has been studied and applied to taxonomy and systematics for a long time. Beyond the standard heart shapes depicted in most textbooks, great variations have also been shown (Atkinson 1973; Nayar and Kaur 1971). These variations include the ability to grow indeterminately and branch so that perennial gametophyte clones of considerable size may be produced (Farrar 1990; Dassler 1995). This morphological diversity may relate to the species' mating system and reproductive biology.

Four types of mating system in homosporous pteridophytes have been defined by Klekowski (1979): (1) intragametophytic selfing, (2) intergametophytic selfing, (3) intergametophytic crossing, and (4) intergametophytic mating. Among these mating systems, intragametophytic selfing had been previously suggested as being predominant in homosporous ferns due to the close proximity of antheridia and archegonia on bisexual gametophytes (Klekowski and Baker 1966; Klekowski 1973, 1979) and indeed there are some homosporous ferns which have very high rates of intragametophytic selfing (Crist and Farrar 1983; McCauley et al. 1985; Soltis and Soltis 1986a; Watano and Sahashi 1992). Polyploid species may also favor intragametophytic selfing due to duplicated alleles which mitigate the problem of recessive deleterious allele expression associated with selfing (Klekowski and Baker 1966; Lloyd 1974; Masuyama and Watano 1990). Recently, however, electrophoretic evidence has shown that intergametophytic mating is the most common

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breeding system in homosporous ferns (Haufler and Soltis 1984; Holsinger 1987; Soltis and Soltis 1986b; Soltis and Soltis 1990; Ranker 1992).

Several types of reproductive behavior can reduce the probability of intragametophytic selfing. Asynchronous maturation of male and female gametangia is one way to avoid intragametophytic selfing in bisexual gametophytes (Klekowski 1968, 1969; Masuyama 1974a, b). Possession of an antheridiogen system is another way to facilitate production of unisexual gametophytes, and thus promote intergametophytic mating (Naf et al. 1975; Voeller 1971; Hamilton and Lloyd 1991). Antheridiogen (or germinin, proposed by Voeller 1971) also overcomes the inhibition of spore germination in darkness (Voeller 1971; Schneller et al. 1990). However, except for Polypodium australe (Welling and Haufler 1993), and Aglaomorpha meyeniana (Naf 1966; 1969), Polypodiaceae s.s. has been thought neither to produce its own antheridiogen nor to respond to the antheridiogen of Pteridium aquilinum (L.) Kuhn, the most common antheridiogen known (Voeller 1971; Raghavan 1989). Genetic load is also an obstacle to successful intragametophytic selfing, and the degree of genetic load has been used to measure sporophyte heterozygosity and probable breeding system (Lloyd 1974; Cousens 1988). However, most of the information known about the reproductive biology of ferns is from research on terrestrial ferns which encounter very different environmental events from epiphytic ferns.

Phlebodium aureum (L.) J. Smith, a member of the Polypodiaceae, distributed from Florida, Mexico, and West Indies to Central and South America (Nauman 1993), is a common epiphytic species in South Florida (Lellinger, 1985). The gametophyte morphology of this species has been briefly described (Nayar and Kaur 1971). Naf (1956) reported that gametophytes of P. aureum did not respond to antheridiogen of Pteridium aquilinum, but he did not test whether it produces its own antheridiogen. Sexual expression and fertilization behavior have been studied (Ward 1954), but other reproductive biology has not been
studied. Due to the epiphytic habitat of this species and the nature of its bark and bryophyte substrate, dispersed spores are likely to be effectively more widely separated, and the interaction of the subsequent gametophytes more hindered than in terrestrial species (Dassler 1995). Thus gametophytes of epiphytic species might be more likely to produce their sporophytes through intragametophytic selfing. This paper investigates this issue in P. aureum, addressing the following questions: (1) how are gametangia expressed in this species, (2) how much genetic load exists, (3) do gametophytes produce and/or respond to antheridiogen, (4) through which mating system are sporophytes produced, (5) how does gametophyte morphology relate to the reproductive system.

**Materials and methods**

Materials were collected in August, 1994 from three populations in Florida: Tosatahachee State Preserve (TS), Jonathan Dickinson State Park (JD), and Fakahatch State Preserve (FS). Ten sporophytes from each population were used for isozyme electrophoresis. One individual (Chiou 14342, label as "A") from FS and one (Chiou 14300, label as "B") from JD were used for two sources of isolated-spore and isolated-gametophyte cultures. The former was also used for multigametophyte culture. Since antheridiogen of Pteridium aquilinum (A.) has been well studied (Naf et al. 1975), and gametophytes of Onoclea sensibilis L. have been used to "safely identify antheridium-inducing activity" due to their complete lack of spontaneous antheridium production in young stages (Naf 1956), these two species were also used in this study to assay the activity of antheridiogen of Phlebodium aureum. Spores of Onoclea sensibilis were collected from Marshall County, Iowa, in September, 1994 (Chiou s.n.), and those of Pteridium aquilinum were in part collected in June, 1991 from Iowa City, Iowa (Farrar 91-6-26-27), and in part were kindly sent by Dr. David Wagner, collected from Eugene, Oregon, in August 3, 1994. Voucher specimens of P. aureum were deposited in the Ada Hayden Herbarium (ISC) of the Department of Botany of
Iowa State University. Spores obtained from fertile fronds (Chiou and Farrar 1994) were stored in the refrigerator at about 5°C.

Spores were sown on 1% agar-solidified media which contained Bold's macronutrients (Bold 1957) and Nitsch's micronutrients (Nitsch 1951), and a trace of ferric chloride (Peck 1985). Cultures were maintained under continuous, white fluorescent illumination of 2000-3000 lux. Temperature was maintained between 20-24°C.

Gametophyte morphology was observed under dissecting and compound microscopes. Pictures were either taken by auto-camera or hand-drawn using a drawing tube. Sizes of gametophytes were measured as the width of the widest part. Gametophytes were observed every half month.

To harvest antheridiogen-enriched solution, the following procedure was used. One mg of spores of *P. aureum* and *P. aquilinum* was sterilized with 10% Clorox for 5 minutes and then rinsed with sterilized water. The sterilized spores were then suspended in 2 ml of sterilized water. Two drops of the spore suspension were placed into each of several petri dishes of 8.5 cm diameter containing about 50 ml of agar media. The cultures of *P. aureum* and *P. aquilinum* gametophytes were frozen at 4 months and 2 months old respectively, then thawed and the liquid extract collected. 2% and 10% dilutions of the extract were made by adding 20 drops (ca. 1 ml) of the extract into large petri dishes containing 50 ml of agar medium and the small petri dishes containing 10 ml of agar medium respectively. In addition to these extracts, GA3 (5x10^-5 g/ml) was also used to test antheridiogen response.

Considering the possibility that some antheridiogens might be short-lived (Emigh and Farrar 1977), that the time of harvesting extract might be not proper, or that the harvesting method may destroy antheridiogen activity, three additional experiments were conducted in which agar with mature growing gametophytes was used directly for the assay. In the first experiment, the agar with growing mature gametophytes was cut into 1 x 2 cm blocks. Then
each agar block was turned on its side (turned block), so the gametophytes stood on one side of the agar block. Ten spores were sown on the top of each block, and ten such blocks were put into each of two petri dishes. In the second experiment, agar supporting mature gametophytes was cut into four parts. Each part was placed upside down in another petri dish, and spores were sown on the upside-down agar (inverted block). In the third experiment, some of the gametophytes were removed from cultures to make a 1 cm wide lane (cleared block), and spores were sown on these cleared areas (between mature gametophytes). All gametophyte cultures assayed for presence of antheridiogen were 4 months old and 2 months old for *P. aureum* and *P. aquilinum* respectively.

The preparation of both extracts and blocks with growing gametophytes were used in tests of germination capacity in darkness and promotion of antheridium formation in the light. A complete randomized design was used. The percentage of plants forming antheridia was counted as the number of male plus bisexual plants. Tests were repeated twice in the first experiment (Tables 5, 7) and four times in the second and third experiments (Tables 6, 8). At each observation time, 25 gametophytes were removed from each dish in the first experiment and 12 gametophytes were removed from each dish in the second and third experiments.

Spore germination capability in darkness and the promotion of spore germination in the dark by antheridiogen were tested by wrapping two layers of aluminum foil around spore-sown petri dishes. These wrapped dishes were placed in a dark cabinet, unwrapped and examined one month later. Tests were repeated twice for 2% extracts of *P. aureum* and *P. aquilinum* gametophyte cultures and for turned blocks of these two species. Tests for 10% extracts, inverted blocks, and cleared blocks of these two species were repeated three times. Germination percentage was determined by counting 100 spores. The proportion of male plants was measured as the number of male gametophytes divided by the total number of dark-germinated gametophytes. A complete randomized design was used.
Genetic load was calculated by comparing isolated-spore and isolated-gametophyte cultures with paired-spore and paired-gametophyte cultures. These cultures were grown on "jelly-mold" plates, each with 20 cells. Each cell contained about 6 ml of agar medium. In each plate, a single spore was transferred onto each of 5 cells, and a one-month-old gametophyte which was still asexual was transferred onto each of another 5 cells. Another 10 cells were used as paired-spore and paired-gametophyte controls. Five such plates were separated by transparencies and stacked into transparent plastic vegetable crispers for a total of 50 replicates of each culture type. The light intensity of these cultures were maintained between 1500 lux (the bottom layer) to 3500 lux (the top layer). Plants were watered every two weeks after gametophytes were 4 months old. Sporophytes were determined to have been produced sexually by examination with a compound microscope. Genetic load was measured by counting the percentage of bisexual gametophytes failing to produce sporophytes. The five layers of plates were designated as blocks, and spore-culture vs. gametophyte-culture were designated as a split plot. Since two source of spores were used, a Latin Square was designed. Five treatments were "A", "B" (isolated-cultures of two different sources), "AA", "BB" (paired-cultures of two same sources), and "AB" (paired-cultures of different sources).

For isozyme observation, the grinding method and buffer followed Farrar (1990). Starch-gel electrophoresis and staining were conducted following Ranker et al. (1989). Thirteen enzyme systems were scored, including aconitate hydratase (ACO), adolase (ALD), fructose-biphosphatase (FBP), glutamate oxaloacetate transaminase (GOT), hexokinase (HK), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PGD), phosphoglucose isomerase (PGI), phosphoglucomutase (PGM), shikimate dehydrogenase (SkDH), and triosephosphate isomerase (TPI). Scoring of the isozyme patterns, followed Werth (1989) since P. aureum has been reported as tetraploid (Evans 1963, Nauman 1993), the most anodal region of
activity of each locus pair was given a numeric abbreviation as "1" (e.g. Hk-1). Members of a locus pair were abbreviated with letters, "a" designated to the more anodal region of activity (e.g. Hk-1a vs. Hk-1b). Isozyme data were calculated and analyzed by BIOSYS-1 (Release 1.7; Swofford and Selander 1989).

Results

Morphology

In multispore cultures, the rhizoid was usually the first cell produced on germination (Fig. 1) about 3 days after sowing spores. The basal cell underwent a second division (Fig. 2), resulting in a protonemal cell which subsequently produced a filament by serial divisions (Fig. 3). The length of the filament was usually two to six cells. In addition to the basal cell, rhizoids also emerged from other cells of the filament other than the top cell. A longitudinal division usually occurred at the apical cell and/or the other cells of the filament (Figs. 4, 5). Subsequent divisions and expansion of filament cells formed a broad spathulate prothallus (Fig. 7). An obconical meristematic cell was built at the anterior margin by two oblique divisions at early or later stages of the spathulate prothallus (Figs. 6, 8). A pluricellular meristem replaced the single meristematic cell later (Figs. 9, 16). In some spathulate prothalli, the meristem appeared laterally (Fig. 17). A symmetrical (or nearly) cordate gametophyte formed eventually (Figs. 10, 11).

In addition to the margin, rhizoids also occurred on both dorsal and ventral sides of the midrib as well as on the wings, but on the latter rhizoids were less than on the midrib or on the margin.

After attaining spathulate stages, gametophytes produced unicellular, papillate, and secretory hairs on the margin (Figs. 9, 12). On older gametophytes, hairs also occurred on both dorsal and ventral surfaces of wings and midribs (Figs. 10-11).
Figs. 1-15. Gametophyte development and response to treatments. Figs. 1-12. Gametophyte development in *Phlebodium aureum*. Figs. 1-9. Germination and early development. Fig. 10. Male gametophyte. Fig. 11. Branched hermaphroditic gametophyte. Fig. 12: Marginal unicellular hair. Fig. 13. 2 month old male gametophyte of *P. aureum* growing on an inverted agar block of mature *P. aquilinum* gametophytes. Fig. 14. 2 month old male gametophyte of *O. sensibilis* growing on a turned block of mature *P. aureum* gametophytes. Fig. 15. 2 month old male gametophyte of *O. sensibilis* growing on a cleared-block of mature *P. aureum* gametophytes. Bar = 0.1 mm unless otherwise indicated.
Figs. 16-21. Gametophyte morphology of *Phlebodium aureum* and experimental treatments. Fig. 16. A one month old symmetrical gametophyte. Fig. 17. A one month old asymmetrical gametophyte. Fig. 18. A 4 month old clone. Fig. 19. Two vegetative proliferations on a gametophyte. Fig. 20. A 1.5 month old gametophyte growing on a cleared block of mature *P. aquilinum* gametophytes. Fig. 21. A 1.5 month old gametophyte growing on an inverted block of mature *P. aquilinum* gametophytes. Bar = 1 cm for Fig. 18; bar = 0.1 mm for Figs. 16, 17, 19-21.
At about 1.5 months, archegonia appeared on a thickened midrib behind the meristem. Antheridia did not appear until at about 2.5 months, usually on the posterior part or sometimes located near the apical meristem if the midrib had not formed (Fig. 10), or mixed with archegonia as gametophytes grew older.

As gametophytes grew older, vegetative propagation, which grew from one or a few cells, occurred on the margin and both surfaces (Fig. 11). The gametophytes continued growing over one year and formed a relatively large mat (Fig. 19). Several sporophytes were derived from one single gametophyte which still remained growing after sporophyte development.

**Sex sequence**

The gametangia did not appear until 1.5 months after sowing spores. Sexual frequency varied with the culture time (Table 1). Archegonia developed first and female plants were most common before the third month. Hermaphrodites appeared at 2 months, probably derived from female plants, and became predominant at 3 months old. Males were relatively small and seemed to be developing from slow-glowing individuals, perhaps from late-germinated spores.

Among isolated-cultures, sex expression was not significantly different either between two sources or among different plate layers (data not shown). In addition, both the isolated-spore and isolated gametophyte cultures had 97% of bisexual and 3% of female at 8 months after spores sown (Table 2).

**Genetic load**

As shown in table 3, the percentage of sporophyte production was not significantly different among treatments, except that fewer sporophytes were produced in the isolated-spore culture of "A". The genetic load was low, 11% on average, and there was no significant
Table 1. The sexual frequency (%) and gametophyte size of *P. aureum* at different times in multi-spore cultures.

<table>
<thead>
<tr>
<th>Width (mm)</th>
<th>1.5 month</th>
<th>2 month</th>
<th>2.5 month</th>
<th>3 month</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A M F H*</td>
<td>A M F H</td>
<td>A M F H</td>
<td>A M F H</td>
</tr>
<tr>
<td>0.5</td>
<td>6 - - -</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
</tr>
<tr>
<td>1</td>
<td>10 - - -</td>
<td>2 - - -</td>
<td>6 - - -</td>
<td>2 2 - -</td>
</tr>
<tr>
<td>2</td>
<td>19 - 2 -</td>
<td>10 - - -</td>
<td>13 2 - -</td>
<td>4 - - -</td>
</tr>
<tr>
<td>3</td>
<td>15 - 25 -</td>
<td>11 - 29</td>
<td>2 2 15 4</td>
<td>- 2 6 8</td>
</tr>
<tr>
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<td>- - 19 -</td>
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<td>- - 31 4</td>
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<td>- - 4 -</td>
<td>- - 8 -</td>
<td>- - 15 4</td>
<td>- - 5 19</td>
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<td>6</td>
<td>- - - -</td>
<td>- - -</td>
<td>- - 2 -</td>
<td>- - - 6</td>
</tr>
<tr>
<td>Total</td>
<td>50 - 50 -</td>
<td>23 - 60</td>
<td>17 21 4 63</td>
<td>12 6 4 17</td>
</tr>
</tbody>
</table>

* A = asexual, M = male, F = female, H = hermaphrodite.

Table 2. The sex expression (%) of *P. aureum* in the isolated-spore (spore) and isolated-gametophyte (gametophyte) culture at 8-month after sowing spores.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Male</th>
<th>Female</th>
<th>Bisexual</th>
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</thead>
<tbody>
<tr>
<td>Spore</td>
<td>0</td>
<td>3</td>
<td>97</td>
</tr>
<tr>
<td>Gametophyte</td>
<td>0</td>
<td>3</td>
<td>97</td>
</tr>
</tbody>
</table>

* Spore and gametophyte cultures are not significantly different in Duncan's multiple test (95% c.l.).

difference either between two sources or between isolated-spore cultures and isolated-gametophyte cultures.

**Isozyme analysis**

Twenty two putative duplicated locus pairs were scored among the thirteen enzyme systems. There was no variability within or among the three populations for 21 locus pairs. Eleven locus-pairs (Ald-a/b, Fbp-1a/b, Fbp-2a/b, Idh-1a/b, Idh-3a/b, Mdh-1a/b, Mdh-3a/b, 6pgd-2a/b, Pgi-1a/b, Skd-1a/b, and Skd-2a/b) were fixed at the same allele, and ten locus-pairs (Aco-1a/b, Aco-2a/b, Hk-a/b, Lap-a/b, Mdh-2a/b, 6Pgd-3a/b, Pgi-2a/b, Pgm-1a/b, Pgm-2a/b, and Tpi-2a/b) had fixed interlocus heterozygosity. The locus-pair Got-a/b displayed
Table 3. The percentage of sporophyte production and genetic load of 8-month-old *P. aureum* in spore-cultures (spore) and gametophyte-cultures (gametophyte) from two sources.

<table>
<thead>
<tr>
<th>Source</th>
<th>% sporophyte</th>
<th>load</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spore</td>
<td>gametophyte</td>
<td>average</td>
<td>spore</td>
</tr>
<tr>
<td>A</td>
<td>77 (b)</td>
<td>82 (ab)</td>
<td>79 (b)</td>
<td>18 (a)</td>
</tr>
<tr>
<td>AA</td>
<td>92 (ab)</td>
<td>90 (ab)</td>
<td>91 (ab)</td>
<td>-</td>
</tr>
<tr>
<td>AB</td>
<td>98 (ab)</td>
<td>80 (ab)</td>
<td>88 (ab)</td>
<td>-</td>
</tr>
<tr>
<td>BB</td>
<td>89 (ab)</td>
<td>100 (a)</td>
<td>94 (a)</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>92 (ab)</td>
<td>97 (ab)</td>
<td>95 (a)</td>
<td>7 (a)</td>
</tr>
</tbody>
</table>


2, 3, 4, 5 The same letter in parentheses indicates no significant difference in Duncan’s multiple test (95% c.l.). SEM = 7.77, 4.59, 7.89, and 6.23 for 2, 3, 4, and 5 respectively.

fixed interlocus heterozygosity within and between TS and JD populations and fixed within 90% of the samples of FS but fixed at the same allele for 1b in the other 10% (Table 4).

The geneotypes between the population TS and JD were exactly the same and the genetic similarity between population FS and the above two populations was high, 0.998 in the Rogers’ genetic coefficient (1972) and and no difference in the Nei’s (1978) genetic coefficient. In the population TS and JD, there was only one genotype for all the locus-pairs. In the population FS, only two genotypes appeared in the only one variable locus-pair Got-a11/b11 (10%) and Got-a11/b22 (90%).

**Spore germination in darkness**

Spores of *P. aureum* did not germinate in the dark control or in any dark treatments.

**Antheridium promotion in Onoclea sensibilis**

Antheridium formation by gametophytes of *O. sensibilis* was not significantly promoted when spores were sown 1.5 months on medium containing 2% and 10% extracts of *P. aureum* gametophyte cultures, but was significantly promoted when spores were sown on
Table 4. Allele frequencies in three populations of *P. aureum*. The sample size in each population is 10.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele</th>
<th>TS*</th>
<th>JD*</th>
<th>FS*</th>
<th>Locus</th>
<th>Allele</th>
<th>TS*</th>
<th>JD*</th>
<th>FS*</th>
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</thead>
<tbody>
<tr>
<td>Aco-1a</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>Mdh-2a</td>
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<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
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<tr>
<td>Aco-1b</td>
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<td>1.00</td>
<td>1.00</td>
<td>Mdh-2b</td>
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<td></td>
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</tr>
</tbody>
</table>

* TS = Tosohatchee State Reserve, JD = Jonathan Dickson State Park, FS = Fakahatchee Strand State Preserve.

turned, inverted, and cleared blocks of mature *P. aureum* gametophytes 1 month after sowing spores (Tables 5, 6). Antheridia were produced from spathulate to early cordate stages (Figs. 14-15). Usually a few antheridia were produced at younger stages but antheridia were abundant on relatively mature gametophytes and active sperms were released.

**Antheridium promotion in Phlebodium aureum**

No treatments promoted antheridium onset during 3 months of culture except when spores of *P. aureum* were sown on agar blocks growing living gametophytes of *P. aquilinum* (Tables 7, 8), and antheridia were not significantly promoted on turned blocks of *P.*
Table 5. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0.5 months</td>
<td>1 month</td>
<td>1.5 months</td>
<td></td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (c)**</td>
<td>2 (b)</td>
<td>10 (b)</td>
<td></td>
</tr>
<tr>
<td>2. Pa 2% extract</td>
<td>0 (a)</td>
<td>0 (b)</td>
<td>15 (b)</td>
<td></td>
</tr>
<tr>
<td>3. Pa turned block</td>
<td>0 (a)</td>
<td>74 (a)</td>
<td>100 (a)</td>
<td></td>
</tr>
</tbody>
</table>

* Pa = *P. aureum.*
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 0.00, 7.07, and 12.73 for 0.5 months, 1 month, 1.5 months respectively, for conditions 2 & 3.

Table 6. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 months</td>
<td>1 month</td>
<td>1.5 months</td>
<td></td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (a)**</td>
<td>6 (b)</td>
<td>17 (b)</td>
<td></td>
</tr>
<tr>
<td>2. Pa 10% extract</td>
<td>0 (a)</td>
<td>4 (b)</td>
<td>13 (b)</td>
<td></td>
</tr>
<tr>
<td>3. Pa cleared block</td>
<td>3 (a)</td>
<td>77 (a)</td>
<td>92 (a)</td>
<td></td>
</tr>
<tr>
<td>4. Pa inverted block</td>
<td>13 (a)</td>
<td>94 (a)</td>
<td>100 (a)</td>
<td></td>
</tr>
</tbody>
</table>

* Pa = *P. aureum.*
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 7.22, 8.86, and 4.21 for 0.5 months, 1 months, and 1.5 months respectively, for conditions 2-4.
Table 7. Antheridium formation (%) by *Phlebodium aureum* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
<th>1.5 months</th>
<th>2.5 months</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td></td>
<td>0 (a)**</td>
<td>0 (a)</td>
<td>4 (b)</td>
</tr>
<tr>
<td>2. Pa 2% extract</td>
<td></td>
<td>0 (a)</td>
<td>4 (a)</td>
<td>8 (b)</td>
</tr>
<tr>
<td>3. Pt 2% extract</td>
<td></td>
<td>4 (a)</td>
<td>0 (a)</td>
<td>8 (b)</td>
</tr>
<tr>
<td>4. Pa turned block</td>
<td></td>
<td>4 (a)</td>
<td>4 (a)</td>
<td>4 (b)</td>
</tr>
<tr>
<td>5. Pt turned block</td>
<td></td>
<td>12 (a)</td>
<td>10 (a)</td>
<td>100 (a)</td>
</tr>
</tbody>
</table>

* Pa = *P. aureum, Pt = *P. aquilinum.
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 2.83, 3.00, and 2.53 for 1.5 months, 2.5 months, and 3 months respectively, for conditions 2-5.

Table 8. Antheridium formation (%) by *Phlebodium aureum* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
<th>1.5 months</th>
<th>2.5 months</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td></td>
<td>2 (b)**</td>
<td>13 (b)</td>
<td>13 (b)</td>
</tr>
<tr>
<td>2. GA3 5x10⁻⁵ g/ml</td>
<td></td>
<td>6 (b)</td>
<td>11 (b)</td>
<td>15 (b)</td>
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<tr>
<td>3. Pa 10% extract</td>
<td></td>
<td>10 (b)</td>
<td>10 (b)</td>
<td>15 (b)</td>
</tr>
<tr>
<td>4. Pt 10% extract</td>
<td></td>
<td>8 (b)</td>
<td>15 (b)</td>
<td>15 (b)</td>
</tr>
<tr>
<td>5. Pa cleared block</td>
<td></td>
<td>0 (b)</td>
<td>0 (b)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>6. Pa inverted block</td>
<td></td>
<td>0 (b)</td>
<td>0 (b)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>7. Pt cleared block</td>
<td></td>
<td>73 (a)</td>
<td>89 (a)</td>
<td>100 (a)</td>
</tr>
<tr>
<td>8. Pt inverted block</td>
<td></td>
<td>84 (a)</td>
<td>98 (a)</td>
<td>98 (a)</td>
</tr>
</tbody>
</table>

* Pa = *P. aureum, Pt = *P. aquilinum.
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 9.11, 5.12, and 4.17 for 1.5 months, 2.5 months, and 3 months respectively, for conditions 3-8.
aquilinum gametophytes until 3 months after sowing spores (Table 7). Antheridia were produced from filaments or from spathulate through young cordate stages of prothallus development (Figs. 13, 20, 21). Active sperms were released from mature antheridia.

Discussion

The morphology and development of Phlebodium aureum gametophytes have been previously described as cordate and as Drynaria-type in development (Nayar and Kaur 1971). In this observation, not only the Drynaria-type but also the Ceratopteris-type of development was occasionally found. Cordate gametophytes also became branched and clone-forming in later development. A clone may benefit this species by increasing the gametophyte's living space and prolonging its life span. These extended clonal and perennial gametophytes continuously form gametangia on their new proliferations, and thus enhance the possibility of interaction with other gametophytes which may establish previously or later.

Intergametophytic mating has been recently thought to be the most common breeding system in homosporous ferns, and genetic load has been suggested to be the primary mechanism controlling outcrossing (Masuyama 1979, 1986; Haufler et al. 1990; Haufler and Welling 1994). In this study, the genetic load is low for gametophytes of both source "A" (average 16%), and source "B" (average 5%). This suggests that sporophytes of P. aureum can be derived through intragametophytic selfing. The conclusion that the low level of genetic load and the intragametophytic mating is the characteristics of pioneer species (Lloyd 1974) is partly supported by P. aureum which is a common epiphyte in greenhouses, gardens, and disturbed forests as well as mature forests.

Masuyama (1979), Masuyama et al. (1987), and Masuyama and Watano (1990) demonstrated that diploid species favored intergametophytic mating whereas tetraploid species favored intragametophytic mating. Both of diploid, n = 37 (Evans 1963, unknown origin), and tetraploid, n = 74 (Evans 1963, Florida origin) have been reported in P. aureum.
Electrophoretic patterns for *P. aureum* revealed high levels of fixed interlocus heterozygosity, indicating that these samples from Florida are polyploid, probably allopolyploid. Because of this, accurate counts of heterozygous individuals and estimates of outcrossing are not possible, but polyploidy and two genetic load in this species indicate probable intragametophytic selfing. The high level of genetic identity among those Florida populations suggests that they may share a common and recent evolutionary history.

Antheridiogen has been considered to be one of the mechanisms promoting outcrossing (Haufler and Welling 1994). In *P. aureum* gametophytes, antheridium onset was not promoted by extracts of *P. aureum* gametophyte cultures, or secretions of living gametophytes of *P. aureum*. Also, antheridium formation in *O. sensibilis* was not promoted by extracts of *P. aureum* gametophyte cultures although it was significantly promoted by secretions of living gametophytes of *P. aureum*. These results indicate that *P. aureum* in our cultures produced only very little antheridiogen (*Apha*) which is insufficient for *P. aureum* to respond but sufficient for *O. sensibilis*. The results that extracts of *P. aquilinum* gametophyte cultures did not promote antheridium formation in *P. aureum* gametophytes but secretions of living gametophytes of *P. aquilinum* did, indicate that *P. aureum* gametophytes need a very strong concentration of antheridiogen of *P. aquilinum* (*Apq*) to respond. The reason that *P. aureum* has been considered to have no response to *Apq* (Naf 1956) is possibly due to using an insufficient concentration of *Apq* or due to too short observation time (9 weeks). In the same paper, Naf (1956) also briefly mentioned "this fern does form antheridia both spontaneously and in response to added antheridial factor, if provided with a more suitable environment". Thus antheridiogen response seems to be related to growth habitat.

Another indirect evidence of antheridiogen *Apha* comes from the male expression in multi-spore cultures, where males were smaller and antheridia were produced later than archegonia (Table 1). This fits the model of antheridiogen function proposed by Naf (1963).
who suggested the fast-growing females secrete antheridiogen which induces slow-growing gametophytes to produce antheridia. Ward (1954), however, reported that *P. aureum* produced antheridia followed by archegonia and favored cross-fertilization in his multispore cultures. On the other hand, he also stated "this development is directly subject to variations induced by a varied environment". This study and the results of Ward (1954) and Naf (1956) indicate that environment may affect the production and response of antheridiogen which affects sexual expression.

Dopp (1950, cited from Naf 1963) found that antheridium extracts also retarded gametophyte growth except at very low concentrations. In this experiment, similarly, all gametophytes growing in the treatment cultures were smaller than those in untreated cultures (data not shown). In treatment cultures, gametophytes derived from those spores sown near other mature gametophytes (higher concentration of active substance) were smaller than those growing on medium containing extract (lower concentration of active substance). We here propose two possible reasons to explain the smaller gametophytes. One possibility is that the potential vegetative growth is diverted to antheridium production as demonstrated by Naf (1956). Although this can explain the stronger effect on small male gametophytes growing near other mature gametophytes of *P. aquilinum*, it does not explain why asexual gametophytes growing in treatment conditions were of similar sizes to male gametophytes. Another possibility is that an inhibitor substance is secreted from the mature gametophytes as shown in some species of *Dryopteris* (Bell 1958). To test this, we sowed spores of *P. aureum* on media treated as in conditions 5 and 6 in the table 8, but using mature gametophytes of *Anemia phyllitidis* instead of *P. aureum* as the antheridiogen source. *A. phyllitidis* is known to produce another type of antheridiogen (AAn) which induces antheridium production in *Anemia* but is inactive in families other than the Schizaeaceae (Naf *et al.* 1975). The size of *P. aureum* in these cultures remained very small, similar in size to the precocious male
gametophytes growing on agar blocks of mature *P. aquilinum* gametophytes, but all remain asexual during the 3 months culture (data not shown). This result indicates that inhibitors may be universal and closely related among fern gametophytes, unlike antheridiogens which are more specific. It seems that *P. aureum* gametophytes respond to the inhibitor and not to the antheridiogen produced by *Anemia phyllitidis*. On the other hand, *P. aureum* responds to both the antheridiogen and inhibitor produced by *P. aquilinum*. An alternative hypothesis is that antheridiogen functions in both antheridium promotion and gametophyte growth inhibition, but the efficiency of these two functions is different depending on the species. The statement that "antheridiogens actually affect size, and size influences sex expression" (Korpelainen 1994), is questioned here, at least this species.

In the Polypodiaceae, neither antheridiogen presence nor response to *Apt* has been thought to exist (Voeller 1971; Raghavan 1989), except for a very weak response in *Aglamorpha meyeniana* (Naf 1966; 1969). However, Welling and Haufler (1993) recently demonstrated that gametophytes of *Polypodium australe* produce antheridiogen. In *P. aureum*, the existence of a weak antheridiogen response may be a vestige from its diploid ancestors where it was of greater significance. The response to antheridiogen of other species, *e.g.*, *Apt*, or to its own antheridiogen under other environment conditions may occasionally occur and reduce the probability of intragametophytic selfing by increasing the number of male plants.

In summary, gametophytes of *P. aureum*, an epiphytic fern of the New World, are probably able to survive perennially through branching. The low level of genetic load indicates that sporophytes are mostly likely produced through intragametophytic selfing. Multiple bands and fixed interlocus heterozygosity at most enzyme loci indicates polyploidy which may favor intragametophytic selfing. The weak antheridiogen system may be a vestige in the tetraploid of *P. aureum*. The clone-forming habit and the prolonged life span may
increase chances to interact with other gametophytes and occasionally outcross. The high level of isozyme similarity within and among all populations suggests that *P. aureum* populations in Florida share a common and recent history.

**acknowledgments**

We thank Dr. Paul N. Hinz for help with statistic analysis, the Florida Department of Environmental Protection for giving permission to collect specimens in Florida, Dr. Roger L. Hammer for providing useful information for collection, Mr. Keith Fisher for help with locating material in Tosohatchee State Reserve, Dr. J. B. Miller and Mr. R. E. Roberts for help with collecting material in Dickinson State Park, Mr. M. Owen for assistance in collecting material in Fakahatchee Strand State Preserve, Mrs. Ming-Ren Huang for helping with plant cultures, data recording, and manuscript preparation. This research was supported by the Department of Botany, Iowa State University, and by the National Science Council of Taiwan, Republic of China.

**Literature cited**


6. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF \textit{PHYMATOSORUS SCOLOPENDRIA} (N. L. BURM.) PICHI. SERM.

This paper is prepared for publication in American Fern Journal

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Department of Botany, Iowa State University, Ames, IA 50011

\textbf{Introduction}

The morphology of fern gametophytes has been studied and applied to taxonomy and systematics for a long time. Beyond the standard heart shapes depicted in most textbooks, great variations have also been shown (Atkinson 1973; Nayar & Kaur 1971). These variations include the ability to grow indeterminately and branch so that perennial gametophyte clones of considerable size may be produced (Farrar 1990; Dassler 1995). The morphological diversity may relate to the species' mating system and reproductive biology.

Four types of mating system in homosporous pteridophytes have been defined by Klekowski (1979): (1) intragametophytic selfing, (2) intergametophytic selfing, (3) intergametophytic crossing, and (4) intergametophytic mating. Among these mating systems, intragametophytic selfing has been previously suggested as being predominant in homosporous ferns due to the close proximity of antheridia and archegonia on bisexual gametophytes (Klekowski & Baker 1966; Klekowski 1973, 1979) and indeed there are some homosporous ferns which have very high rates of intragametophytic selfing (Crist & Farrar 1983; McCauley \textit{et al.} 1985; Soltis & Soltis 1986a; Watano & Sahashi 1992). Polyploid species may also favor intragametophytic selfing due to duplicated alleles which mitigate the problem of recessive deleterious allele expression associated with selfing (Klekowski & Baker 1966; Lloyd 1974a; Masuyama & Watano 1990). Recently, however, electrophoretic evidence has shown that intergametophytic mating is the most common breeding system in

Several types of reproductive behavior can reduce the probability of intragametophytic selfing. Asynchronous maturation of male and female gametangia is one way to avoid intragametophytic selfing in bisexual gametophytes (Klekowski 1968, 1969; Masuyama 1974a, b). Possession of an antheridiogen system is another way to facilitate production of unisexual gametophytes, and thus promote intergametophytic mating (Naf et al. 1975; Voeller 1971; Hamilton & Lloyd 1991). Antheridiogen (or germinin, proposed by Voeller 1971) also overcomes the inhibition of spore germination in darkness (Voeller 1971; Schneller et al. 1990). However, except for Polypodium australe (Welling & Haufler 1993), and Aglaomorpha meyeniana (Naf 1966; 1969), Polypodiaceae s.s. has been thought neither to produce its own antheridiogen nor to respond to the antheridiogen of Pteridium aquilinum, the most common antheridiogen known (Voeller 1971; Raghavan 1989). Genetic load is also an obstacle to successful intragametophytic selfing, and the degree of genetic load has been used to measure the sporophyte heterozygosity and probable breeding system (Lloyd 1974 a; Cousens 1988). However, most of the information known about the reproductive biology of ferns is from research on terrestrial ferns which encounter very different environmental events from epiphytic ferns.

*Phymatosorus scolopendria*, a member of Polypodiaceae, is terrestrial but often climbs rocks or trunks by its long rhizome. Occasionally it is also epiphytic in large limbs. The gametophyte morphology of this species has been previously studied (Pal & Pal 1962). The mating system has also been reported (Lloyd 1974b), but its other reproductive biology has not been studied. Gametophytes of several epiphytic species of Polypodiaceae have been shown to be perennial and clone-forming (Chapters 1-5). These traits have been suggested to be an adaptation to the epiphytic habitat, enhancing the probability of
gametophyte interaction in an environment (bryophyte mats) more challenging to intergametophytic mating than the terrestrial environment (Dassler 1995). Whether these traits are maintained in hemiepiphytic Polypodiaceae is unknown. This paper will investigate this issue in *Phymatosorus scolopendria*, addressing the following questions: (1) how are gametangia expressed in this species, (2) how much genetic load exists, (3) do gametophytes produce or/and respond to antheridiogen, (4) through which mating system are sporophytes produced, (5) how does gametophyte morphology relate to the reproductive system.

**Materials and methods**

Spores of *P. scolopendria* were collected in August, 1992 from Hawaii (*Farrar 92-8-20-1*) for multispore cultures and antheridiogen assay, and in August, 1994 from Selby Botanical Garden, Sarasota, Florida (*Chiou 14338*) for isolated-and paired-cultures. Since antheridiogen of *Pteridium aquilinum* (L.) Kuhn (Apt) has been well studied (Naf *et al.* 1975), and gametophytes of *Onoclea sensibilis* L. have been used to "safely identify antheridium-inducing activity" due to their complete lack of spontaneous antheridium production in early stages (Naf 1956), these two species were also used in this study to assay the activity of antheridiogen of *P. scolopendria*. Spores of *Onoclea sensibilis* were collected from Marshall County, Iowa, in September, 1994 (*Chiou s.n.*), and those of *Pteridium aquilinum* were in part collected in June, 1991 from Iowa City, Iowa (*Farrar 91-6-26-27*), and in part were kindly sent by Dr. David Wagner, collected from Eugene, Oregon, in August 3, 1994. Voucher specimens of *P. scolopendria* were deposited in the Ada Hayden Herbarium (ISC) of the Department of Botany in Iowa State University. Spores obtained from fertile fronds (*Chiou & Farrar 1994*) were stored in the refrigerator at about 5°C.

Spores were sown on 1% agar-solidified media which contained Bold's macronutrients (Bold 1957) and Nitsch's micronutrients (Nitsch 1951), and a trace of ferric
chloride (Peck 1985). Cultures were maintained under continuous, white fluorescent illumination of 2000-3000 lux. Temperature was maintained between 20-24°C.

Gametophyte morphology was observed under dissecting and compound microscopes. Pictures were either taken by auto-camera or hand-drawn using a drawing tube. Sizes of gametophytes were measured as the width of the widest part. Gametophytes were observed every half month.

To harvest antheridiogen-enriched solution, the following procedure was used. One mg of spores of *P. scolopendria* and *P. aquilinum* was separately sterilized with 10% Clorox for 5 minutes and then rinsed. The sterilized spores were then suspended in 2 ml of sterilized water. Two drops of the spore suspension were placed into each of several petri dishes of 8.5 cm diameter containing about 50 ml of agar media. The cultures of *P. scolopendria* and *P. aquilinum* gametophytes were frozen at 4 months and 2 months old respectively, then thawed and the liquid extract collected. 2% and 10% dilutions of the extract were made by adding 20 drops (ca. 1 ml) of the extract into large petri dishes containing 50 ml of agar medium and the small petri dishes containing 10 ml of agar medium respectively. In addition to these extracts, GA3 (5x10^{-5} g/ml) was also used to test antheridiogen response.

Considering the possibility that some antheridiogens might be short-lived (Emigh & Farrar 1977), that the time of harvesting extract might be not proper, or that the harvesting method may destroy antheridiogen activity, three additional experiments were conducted in which agar with mature growing gametophytes was used directly for the assay. In the first experiment, the agar with growing mature gametophytes was cut into 1 x 2 cm blocks. Then each agar block was turned on its side (turned block), so the gametophytes stood on one side of the agar block. Ten spores were sown on the top of each block, and ten such blocks were put into each of two petri dishes. In the second experiment, agar supporting mature gametophytes was cut into four parts. Each part was placed upside down in another petri
dish, and spores were sown on the upside-down agar (inverted block). In the third experiment, some gametophytes were removed from cultures to make a 1 cm width lane (cleared block), and spores were sown on these cleared areas (between mature gametophytes). All gametophyte cultures assayed for antheridiogen were 4 months and 2 months old for *P. scolopendria* and *P. aquilinum* respectively. A complete randomized design was used. Preparations of both extracts and blocks with growing gametophytes were used in tests of germination capacity in darkness and promotion of antheridium formation in the light. The percentage of plants forming antheridium was counted as the number of male plus bisexual individuals. Tests were repeated twice in the first experiment (Tables 5, 7) and four times in the second and third experiments (Tables 6, 8). At each observation time, 25 gametophytes were removed from each dish in the first experiment and 12 gametophytes were removed from each dish in the second and third experiments.

Capability of spore germination in darkness and the promotion of spore germination in the dark by antheridiogen were tested by wrapping two layers of aluminum foil around spore-sown petri dishes. These wrapped dishes were placed in a dark cabinet and unwrapped and examined one month later. Tests were repeated 2 (table 3) and 3 (table 4) times. Germination rates were determined by counting 100 spores; the proportion of male plants was measured as the number of male gametophytes divided by the total number of dark-germinated gametophytes. A complete randomized design was used.

Genetic load was calculated by comparing isolated-spore and isolated-gametophyte cultures with paired-spore and paired-gametophyte cultures. These cultures were grown on "jelly-mold" plates, each with 20 cells. Each cell contained about 6 ml of agar medium. In each plate, a single spore was transferred onto each of 5 cells, and a one-month-old gametophyte which was still asexual was transferred onto each of another 5 cells. Another 10 cells were used as paired-spore and paired-gametophyte controls. Five such plates were
separated by transparent plastic sheets and stacked into transparent plastic crispers for a total of 50 replicates of each culture type. The light intensity was maintained between 1500 lux (the bottom layer) and 3500 lux (the top layer). Plants were watered every two weeks after gametophytes were 4 months old. Genetic load was measured by counting the percentage of bisexual gametophytes failing to produce sporophytes. The five layers of plates were designated as blocks, and spore- vs. gametophyte-culture were designated as a split plot.

Results

Morphology

In multispore cultures, spores began to germinate the second day after sowing spores, but germination was delayed about one week when spores were stored in the refrigerator at about 5° for more than six months. Spores maintained their viability at 77% in average after 2 years of storage. After the basal cell, the rhizoid was usually the first cell produced on germination (Fig. 1). The basal cell underwent a second division (Fig. 2), resulting in a protonemal cell which subsequently produced a filament by serial divisions (Fig. 3). The lengths of filaments varied from two to six cells. In addition to the basal cell, rhizoids also emerged from other cells of the filament below the top cell (Figs. 6, 7).

About one week after germination, a longitudinal division usually occurred at the apical cell and/or cells bellow the top cell (Figs. 4, 5), and a subsequent spathulate prothallus developed through apical growth (Fig. 6). An obconical meristematic cell was built at the anterior margin by two oblique divisions later (Fig. 7).

A pluricellular meristem replaced the single meristematic cell later, and a nearly symmetrical cordate gametophyte formed finally (Figs. 8-10). The anterior part of some older gametophytes twisted into the shape of cornucopia (Fig. 11). Branches arising from one or a few cells occurred on older gametophytes (Fig. 12). A mat of overlapping branches thus formed (Fig. 31). Gametangia continuously formed on the new proliferations. Gametophytes
Figs. 1-18. Gametophyte development in *Phymatosorus scolopendria*. Figs. 1-7. Germination and early development. Fig. 8. A female gametophyte. Fig. 9. A male gametophyte. Figs. 10-11. Hermaphroditic gametophytes. Fig. 12. A 4 month old hermaphroditic gametophyte with several proliferations. Fig. 13-16. Marginal hairs. Figs. 17, 18. Surface hairs. An = antheridium. Ar = archegonium. Mh = multicellular hair. Uh = unicellular hair.
continued growing over two years and several sporophytes were derived from many of the individual gametophytes, which still remained growing after sporophyte production (Fig. 32).

In addition to the margin, rhizoids also occurred on both dorsal and ventral sides of the midrib as well as on the wings, but those on the latter were fewer than those on the midrib or on the margin. Occasionally, the rhizoid forked (Fig. 7).

After attaining a later stage of spathulate or cordate shape, gametophytes produced hairs most of which were unicellular (Fig. 13), glandular, and appeared on the margins first, then on both dorsal and ventral sides of wings and midribs. Multicellular branched or unbranched hairs (Figs. 14-18) also occurred on the margin as well as on both surfaces.

At about 1.5 months, both antheridia and archegonia appeared. The former were usually on the posterior part (Figs. 10-12), or sometimes near the apical meristem if a midrib had not formed (Fig. 9). Archegonia were distributed on the midrib behind the meristem (Figs. 8, 10-12). Antheridia and archegonia were found either on the ventral or the dorsal side or both.

**Sex sequence**

In multispore cultures, sexual frequency varied with the culture time (Table 1). Male and female plants occurred at almost the same time, but the latter became predominant later while the former remained relatively infrequent during the 3-month culture and seemed to be developing from slowing individuals, perhaps late-germinated spores. Hermaphrodites derived last and gradually became the principal form after 4 months (data not shown). The sizes of hermaphroditic gametophytes were between the males and females (Table 1, Figs. 10, 11), and hard to tell which one they evolved from, perhaps from both.

In isolated cultures, the percentage of sex expression was not significantly different either among different plate layers or between the spore- and gametophyt-culture. All of them became bisexual when they were one year old (data not shown).
Figs. 19-30. Gametophyte response to experimental treatments. Figs. 19-28. *Phymatosorus scolopendria*. Figs. 19, 20. Dark-growing gametophytes on the media containing 10% extract of *Pteridium aquilinum* gametophytes. Figs. 21, 22. Dark-growing gametophytes on an inverted block of *Pteridium aquilinum* gametophytes. Fig. 23. Dark-growing gametophyte on an inverted block of *P. scolopendria* gametophytes. Fig. 24. 1.5 month old gametophyte growing on a cleared block of *P. aquilinum* gametophytes. Fig. 25. 2 month old gametophyte growing on an inverted block of *P. aquilinum* gametophyte. Fig. 26. 1.5 month old gametophyte growing on an inverted block of *P. scolopendria* gametophytes. Fig. 27. 1.5 month old gametophyte growing on a cleared block of *P. scolopendria* gametophytes. Fig. 28. 2.5 month old gametophyte growing on the medium containing 10% extract of *P. scolopendria* gametophyte cultures. Figs. 29-30. *Onoclea sensibilis*. Fig. 29. 0.5 month old gametophyte of *Onoclea sensibilis* growing on an inverted block of *P. scolopendria* gametophytes. Fig. 30. A one month old gametophyte of *O. sensibilis* growing on medium containing 10% extract of mature *P. scolopendria* gametophyte cultures. An = antheridium. Bar = 0.1 mm.
Figs. 31-35. Morphology and development of *P. scolopendria* gametophytes and response to dark treatments. Figs. 31-32. gametophyte morphology and development. Fig. 31. A 6 month old clone. Fig. 32. Several sporophytes deriving from an one year old gametophytes. Figs. 33-35. Dark germination in response to treatments. Fig. 33. A dark-growing gametophyte on a cleared block of *P. scolopendria* gametophytes. Fig. 34. A dark-growing gametophyte on an inverted block of *P. aquilinum* gametophytes. Fig. 35. A dark-growing gametophyte on medium containing 10% extract of *P. aquilinum* gametophyte cultures. Bar = 1 cm for Fig. 32; bar = 1 mm for Fig. 31; bar = 0.1 mm for Figs. 33-35.
Table 1. The sexual frequency (%) and gametophyte size of *P. scolopendria* at different ages in the multi-spore cultures.

<table>
<thead>
<tr>
<th>Width (mm)</th>
<th>1.5 months</th>
<th>2 months</th>
<th>2.5 months</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMFH*</td>
<td>AMFH</td>
<td>AMFH</td>
<td>AMFH</td>
</tr>
<tr>
<td>0.5</td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>33</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>-</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>83</td>
<td>8</td>
<td>9</td>
<td>-</td>
</tr>
</tbody>
</table>

* A = asexual, M = male, F = female, H = hermaphrodite.

**Genetic load**

As shown in table 2, *P. scopopendria* is highly successful both in isolated-cultures and in paired-cultures in producing sporophytes at one year after sowing spores. Thus the genetic load was low, 11 in average, and there was no significant difference between isolated-spore and isolated-gametophyte cultures. Sporophyte production were not significantly different among different plate layers (data not shown).

**Spore germination in darkness**

Under darkness, spores of *P. scolopendria* did not germinate in controls, or in treatments where GA3, 2% or 10% culture extracts of *P. scolopendria* gametophytes was added, but germinated on other treatments (Tables 3, 4). Germination of *P. scolopendira* spores in darkness were slightly higher in the treatments of *P. aquilinum* gametophytes than in those of *P. scolopendria* gametophytes (Table 3, conditions 5, 6 vs. conditions 4; Table 4, conditions 5, 6, 8 vs. conditions 3, 4). The effects of the extract of the gametophyte...
Table 2. The percentage of sporophyte production (by surviving sporophytes) and genetic load (by bisexual gametophytes) of 1 year old *P. scolopendria* in spore-cultures (spore) and gametophyte-cultures (gametophyte).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% sporophyte</th>
<th>load</th>
</tr>
</thead>
<tbody>
<tr>
<td>isolated</td>
<td>88(a)(^1)</td>
<td>90(a)(^1)</td>
</tr>
<tr>
<td>paired</td>
<td>93(a)(^1)</td>
<td>93(a)(^1)</td>
</tr>
</tbody>
</table>

\(^1\), \(^2\), \(^3\) The same letter in parentheses means no significant difference in Duncan's multiple test (95% c.l.). SEM = 3.92, 2.35, and 2.51 for \(^1\), \(^2\), and \(^3\) respectively.

cultures of *P. aquilinum* were not significantly different from the secretion of their living gametophytes.

Antheridium promotion for all effective treatments was not significantly different (Tables 3, 4). Most dark-growing gametophytes produced antheridia at very young stages when they possessed only a few prothallial cells (Figs. 19, 21-23, 33-35) which were pale-white and often relatively elongated (Fig. 19). Some of these gametophytes remained asexual (Fig. 20). Many antheridia were mature and released active sperms (Fig. 33).

**Antheridium promotion in *Onoclea sensibilis***

2% extract of *P. scolopendria* gametophyte cultures did not significantly promote antheridium formation in gametophytes of *O. sensibilis* until 1.5 months after sowing spores (Table 5). A 10% extract had a significant effect at 1 month (Table 6), and the secretions of living gametophytes of *P. scolopendria* significantly promoted antheridium formation at 0.5 month (Tables 5, 6). Among effective treatments, the influences of secretions from living gametophytes are greater than those of extracts (Table 5, condition 3 vs. condition 2; Table 6, conditions 3, 4 vs. condition 2). Antheridia were produced from very young filaments or spathulate through heart-shaped stages of prothallus development. Usually only a few antheridia were formed by the young stages (Figs. 30, 38), but there were relatively more on
Table 3. Average dark germination percentage and male percentage of *P. scolopendria* under different conditions (experiment I) at one month after sowing spores.

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Germination %**</th>
<th>Male %**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Light control</td>
<td>82 (a)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>2. Dark control</td>
<td>0 (d)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>3. Ps 2% extract</td>
<td>0 (d)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>4. Ps turned block</td>
<td>11 (c)</td>
<td>94 (a)</td>
</tr>
<tr>
<td>5. Pt 2% extract</td>
<td>31 (b)</td>
<td>91 (a)</td>
</tr>
<tr>
<td>6. Pt turned block</td>
<td>32 (b)</td>
<td>93 (a)</td>
</tr>
</tbody>
</table>

* P.s = *P. scolopendria*, Pt = *P. aquilinum*.

** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 1.48 and 3.25 for germination % and male % respectively, for conditions 3-6.

Table 4. Average dark germination percentage and male percentage of *P. scolopendria* under different conditions (experiment II) at one month after sowing spores.

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Germination %**</th>
<th>Male %**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Light control</td>
<td>77 (a)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>2. Dark control</td>
<td>0 (e)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>3. Ps inverted block</td>
<td>13 (cd)</td>
<td>95 (a)</td>
</tr>
<tr>
<td>4. Ps cleared block</td>
<td>8 (d)</td>
<td>89 (a)</td>
</tr>
<tr>
<td>5. Pt inverted block</td>
<td>18 (bc)</td>
<td>80 (a)</td>
</tr>
<tr>
<td>6. Pt cleared block</td>
<td>19 (bc)</td>
<td>89 (a)</td>
</tr>
<tr>
<td>7. Ps 10% extract</td>
<td>0 (e)</td>
<td>0 (b)</td>
</tr>
<tr>
<td>8. Pt 10% extract</td>
<td>23 (b)</td>
<td>95 (a)</td>
</tr>
<tr>
<td>9. GA3 5x10⁻⁵ g/ml</td>
<td>0 (e)</td>
<td>0 (b)</td>
</tr>
</tbody>
</table>

* Ps = *P. scolopendria*, Pt = *P. aquilinum*.

** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 2.32 and 5.82 for germination % and male % respectively, for conditions 3-8.

later stages (Figs. 29, 39) and active sperms were released.

**Antheridium promotion in *P. scolopendria***

A few gametophytes of *P. scolopendria* produced antheridia either under control conditions or on the media to which GA3 was added, but the percentages of male...
Table 5. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (b)**</td>
</tr>
<tr>
<td>2. Ps. 2% extract</td>
<td>8 (b)</td>
</tr>
<tr>
<td>3. Ps. turned block</td>
<td>70 (a)</td>
</tr>
</tbody>
</table>

* Ps = *P. scolopendria*.
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 3.94, 8.49, and 7.07 for 0.5 months, 1 month, and 1.5 months respectively, for conditions 2-3.

Table 6. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (c)**</td>
</tr>
<tr>
<td>2. Ps 10% extract</td>
<td>0 (c)</td>
</tr>
<tr>
<td>3. Ps cleared block</td>
<td>48 (a)</td>
</tr>
<tr>
<td>4. Ps inverted block</td>
<td>67 (b)</td>
</tr>
</tbody>
</table>

* Ps = *P. scolopendria*.
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 4.47, 4.27, and 1.63 for 0.5 months, 1 month, and 1.5 months respectively, for conditions 2-4.

Gametophytes were significantly less than those of other treatments (Tables 7, 8). 2% extracts of *P. scolopendria* and *P. aquilinum* gametophyte cultures had effects similar to secretions of living gametophytes on turned blocks at 1.5 months but significantly less than the latter eventually (Table 7, conditions 2, 3 vs. conditions 4, 5). On the other hand, 10% extracts of *P. scolopendria* and *P. aquilinum* gametophyte cultures were less promoted antheridium
Table 7. Average antheridium formation (%) by *P. scolopendria* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (b)**</td>
</tr>
<tr>
<td>2. Ps 2% extract</td>
<td>46 (a)</td>
</tr>
<tr>
<td>3. Pt 2% extract</td>
<td>48 (a)</td>
</tr>
<tr>
<td>4. Ps turned block</td>
<td>52 (a)</td>
</tr>
<tr>
<td>5. Pt turned block</td>
<td>50 (a)</td>
</tr>
</tbody>
</table>

*Ps = *P. scolopendria*, Pt = *P. aquilinum*.

**The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 4.24, 3.61, and 8.06 for 1.5 months, 2 months, and 2.5 months respectively, for conditions 2-5.

Table 8. Average antheridium formation (%) by *P. scolopendria* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>8 (c)**</td>
</tr>
<tr>
<td>2. GA3 5x10⁻⁵ g/ml</td>
<td>12 (c)</td>
</tr>
<tr>
<td>3. Ps 10% extract</td>
<td>50 (b)</td>
</tr>
<tr>
<td>4. Pt 10% extract</td>
<td>67 (b)</td>
</tr>
<tr>
<td>5. Ps cleared block</td>
<td>82 (a)</td>
</tr>
<tr>
<td>6. Ps inverted block</td>
<td>88 (a)</td>
</tr>
<tr>
<td>7. Pt cleared block</td>
<td>79 (a)</td>
</tr>
<tr>
<td>8. Pt inverted block</td>
<td>71 (ab)</td>
</tr>
</tbody>
</table>

*Ps = *P. scolopendria*, Pt = *P. aquilinum*.

**The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 8.40, 3.63, and 3.28 for 1.5 months, 2 months, and 2.5 months respectively, for conditions 3-8.
Figs. 36-39. Antheridium formation in response to treatments. Figs. 36-37. *P. scopopendria*. Fig. 36. A gametophyte growing on an inverted block of *P. aquilinum* gametophytes. Fig. 37. A gametophyte growing on a cleared block of *P. scolopendria* gametophytes. Figs. 38-39. *Onoclea sensibilis*. Fig. 37. A *O. sensibilis* gametophyte growing on an inverted block of *P. scolopendria* gametophytes. Fig. 38. A *O. sensibilis* gametophyte growing on the medium containing 2% extract of *P. scolopendria* gametophyte cultures. Bar = 0.1 mm.
formation than secretions of their living gametophytes at 1.5 months, but became nonsignificantly different eventually (Table 8, conditions 3, 4 vs. conditions 5-8). Influence on antheridium formation were not significantly different between by *P. scolopendria* and by *P. aquilinum* under effective treatment conditions. Gametophytes began to produce antheridia at very young age (Figs. 24-27, 36) through spathulate stages (Figs. 28, 37) and remained relatively small, especially in block cultures. Active sperms were released from these precocious male gametophytes (Figs. 36, 37).

**Discussion**

The gametophyte of *P. scolopendria* has been illustrated as cordate shaped (Pal & Pal 1962), and the development as Drynaria type (Nayar & Kaur 1971). Those characteristics are in accordance with this study. However, multicellular hairs have not been reported previously, and many proliferations occurred on the older gametophytes. These may function to increase the space occupied by an individual gametophyte and to prolong its life span. These extended clonal and perennial gametophytes continuously form gametangia on their new proliferations, and thus enhance the possibility of interaction with other gametophytes established previously or later.

Intergametophytic mating has been recently thought the most common breeding system in homosporous ferns (Haufler & Soltis 1984; Soltis & Soltis 1990), and genetic load has been suggested to be the primary mechanism promoting outcrossing (Masuyama 1979, 1986; Haufler et al. 1990; Haufler & Welling 1994). However, the low genetic load in this study is similar to the result of Lloyd (1974b) and suggests sporophytes of *P. scolopendria* can be derived from intragametophytic selfing. The hypothesis that low levels of genetic load and intragametophytic selfing characterize pioneering species (Lloyd 1974a) fits this species.

Masuyama (1979), Masuyama et al. (1987), and Masuyama & Watano (1990) demonstrated that diploid species favored intergametophytic mating whereas tetraploid
species favored intragametophytic selfing. Both diploid (2n = 72, Love et al. 1977) and tetraploid (n = 72, Tsai & Shieh 1983) have been reported in *P. scolopendria*. The latter was sampled from Taiwan, an island at the west side of Pacific, and supports the fact that this species can reproduce through intragametophytic selfing.

Antheridiogen has been considered to be a mechanism promoting outcrossing (Haufler & Welling 1994). For both *Onoclea sensibilis* and *P. scolopendria* gametophytes, antheridium formation was promoted significantly when spores were sown on media where extracts of *P. scolopendria* gametophyte cultures were added, and on media where mature *P. scolopendria* gametophytes grew (Tables 5-8). These results suggest that *P. scolopendria* produce its own antheridiogen (A_{phs}). A_{phs} apparently is not similar to GA3 because the latter did not enhance antheridium formation of this species. Furthermore, due to the response of *P. scolopendria* gametophytes to antheridiogen of *Pteridium aquilinum* (A_{pt}), A_{phs} may be more closely related to A_{pt}.

Another indirect evidence of an antheridiogen system in *P. scolopendria* comes from the pattern of male expression in multi-spore cultures, where male gametophytes are smaller and most antheridia are produced later while female gametophytes are larger and most archegonia develop earlier. This fits the model of the antheridiogen function proposed by Naf (1963) who suggested that fast-growing females secrete antheridiogen which induces slow-growing gametophytes to produce antheridia.

Dopp (1950, cited from Naf 1963) found that the active antheridiogen extract also retarded gametophyte growth except at very low concentration. In this experiment, similarly, all gametophytes growing in the effectively treated cultures were smaller than those in the other cultures (data not shown). In treated cultures, gametophytes derived from spores sown near other mature gametophytes (higher concentration of active substance) were smaller than those growing on medium containing extract (lower concentration of active substance). We
here propose two possibilities to explain the smaller gametophytes. One is that the potential vegetative growth is diverted to antheridium production as demonstrated by Naf (1956). Although this can explain the stronger effect on smaller male gametophytes, it does not explain why asexual gametophytes are of sizes similar to male gametophytes. Another possibility is that some inhibitor substance secreted from the mature gametophytes as shown in some species of *Dryopteris* (Bell 1958). To test this, we sowed spores of *P. scolopendria* on media treated as condition 5 and 6 in the table 8, but using mature gametophytes of *Anemia phyllitis* instead of *P. scolopendria*. *A. phyllitidis* is known to produce another type of antheridiogen (*A*$_{An}$) which induces antheridium production in *Anemia* but inactive in families other than the Schizaeaceae (Naf et al. 1975). The size of *P. scolopendria* in these cultures remained very small, similar in size to the precocious male gametophytes described above, but all remain asexual during the 3-month culture (data not shown). These results indicate that inhibitors may be universal and more closely related than antheridiogens among fern gametophytes. It seems that *P. scolopendria* can produce its own antheridiogen as well as inhibitor, and responds to both of them as well as to those produced by *Pteridium aquilinum*. On the other hand, for *A. phyllitidis*, *P. scolopendria* responds to its inhibitor but not to the antheridiogen *A*$_{An}$. An alternative hypothesis is that antheridiogen functions in both of antheridium production and gametophyte growth inhibition, but the efficiency of these two function is different depending on the species. The statement that "antheridiogens actually affect size, and size influences sex expression" (Korpelainen 1994) is questioned here, at least in this species.

In the Polypodiaceae, neither antheridiogen presence nor response to *A*$_{pt}$ has been thought to exist (Naf 1956; Voeller 1971; Raghavan 1989), except for a very week response in *Aglamorpha meyeniana* (Naf 1966; 1969). Recently, Welling & Haufler (1993) also
demonstrated antheridiogen response in *Polypodium australe*. *P. scolopendria* should be added to the list of Polypodiaceae with an antheridiogen system.

Based on their laboratory results, Schneller *et al.* (1990) suggested a correlation between the antheridiogen response and genetic load. *P. scolopendria* has both antheridiogen and low genetic load. The existence of an antheridiogen response could function to reduce the probability of intragametophytic selfing of gametophytes by increasing the number of male plants. The antheridiogen system here may compensate for some degree of inbreeding by facilitating occasional outcrossing to maintain genetic diversity as noted by Watano & Masuyama (1991) in *Ceratopteris thalictroides*. It is also possible that the low genetic load in *P. scopopendria* reflects a polyploid state. If so, an antheridiogen system may be an unimportant vestige in *P. scolopendria*, but of great significance in its diploid ancestors.

Some substance secreted from the mature gametophytes substitutes for the requirement of light for spore germination in the dark. We do not know if this substance is the antheridiogen *Aphs* or the so-called germinin (Voeller 1971). In general, functions of the substance on promoting antheridium formation and the substance substituting for a light requirement for spore germination are parallel (Voeller 1971). However, Welling & Haufler (1993) hypothesized that these two substances may function independently.

In our experiments, neither 2% nor 10% extract of *P. scolopendria* gametophytes induced spores to germinate under darkness (Tables 3, 4), although they did promote antheridium formation in the light (Tables 7, 8). Several explanations are possible. First, antheridiogen and germinin may be different substances. Germinin may be short-lived, or lose activity during the process of extraction, or is required in high concentrations to promote spore germination in darkness. Thus the extractions, which might contain no active germinin or contain insufficient amounts of germinin, could not induce spore germination in darkness. Second, antheridiogen and germinin may be the same substance, but the threshold
concentration for inducing antheridium formation is different from that for promoting dark germination of spores. Evidence supporting this is the fact that the same treatment concentration induced relatively low percentages of spore germination in darkness whereas it promoted a very high percentage of antheridium production (Table 4, conditions 3, 4; Table 5, conditions 3, 4). However, darkness is also able to cancel a light-dependent antheridium block and thus allow antheridia to form spontaneously (Naf et al. 1974). Without more evidence, we cannot conclude if antheridiogen and germinin is the same or different.

A soil spore bank has been demonstrated to exist for terrestrial species (Dyer & Lindsay 1992). For a hemiepiphytic species, P. scolopendria spores landing on the ground can be buried by soil. Antheridiogen (or germinin) can function in inducing germination of those buried spores. Through the antheridiogen influence, the resultant precocious gametophytes can produce antheridia and release active sperms to interact with older gametophytes. This model has been proposed previously (Voeller 1971; Schneller et al. 1990; Haufler & Welling 1994).

In summary, P. scolopendria, a tropical hemiepiphytic fern, gametophytes are able to survive perennially through vegetative proliferation. Sporophytes can be produced through selfing due to a low genetic load. Antheridiogen (or germinin) can induced germination of buried spores. An antheridiogen system may also function to increase the number of male gametophytes and thus promote some intergametophytic mating, partially compensating for the relatively low genetic variation caused by inbreeding. A clone-forming habit, prolonged life span, and antheridiogen (and germinin?) together provides a mechanism to promote interaction and outcrossing with other gametophytes which may develop from spores either dispersed later or previously buried in the soil. If the tested plants are polyploid, these characteristics may to some degree be vestigial, reflecting inheritance from diploid ancestors.
Acknowledgments

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Literature cited


7. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF POLYPODIUM PELLUCIDUM KAULF.

This paper is prepared for publication in American Fern Journal
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Introduction

The morphology of fern gametophytes has been studied and applied to taxonomy and systematics for a long time. Beyond the standard heart shapes depicted in most textbooks, great variations have also been shown (Atkinson 1973; Nayar and Kaur 1971). These variations include the ability to grow indeterminately and branch so that perennial gametophyte clones of considerable size may be produced (Farrar 1990; Dassler 1995). The morphological diversity may relate to the species' mating system and reproductive biology.

Four types of mating system in homosporous pteridophytes have been defined by Klekowski (1979): (1) intragametophytic selfing, (2) intergametophytic selfing, (3) intergametophytic crossing, and (4) intergametophytic mating. Among these mating systems, intragametophytic selfing had been previously suggested as being predominant in homosporous ferns due to the close proximity of antheridia and archegonia on bisexual gametophytes (Klekowski and Baker 1966; Klekowski 1973, 1979) and indeed there are some homosporous ferns which have very high rates of intragametophytic selfing (Crist and Farrar 1983; McCauley et al. 1985; Soltis and Soltis 1986a; Watano and Sahashi 1992).

Polyploid species may also favor intragametophytic selfing due to duplicated alleles which mitigate the problem of recessive deleterious allele expression associated with selfing (Klekowski and Baker 1966; Lloyd 1974; Masuyama and Watano 1990). Recently, however, electrophoretic evidence has shown that intergametophytic mating is the most common breeding system in homosporous ferns (Haufler and Soltis 1984; Holsinger 1987; Soltis and Soltis 1986b; Soltis and Soltis 1990; Ranker 1992).
Several types of reproductive behavior can reduce the probability of intragametophytic selfing. Asynchronous maturation of male and female gametangia is one way to avoid intragametophytic selfing in bisexual gametophytes (Klekowski 1968, 1969; Masuyama 1974a, b). Possession of an antheridiogen system is another way to facilitate production of unisexual gametophytes, and thus promote intergametophytic mating (Naf et al. 1975; Voeller 1971; Hamilton and Lloyd 1991). Antheridiogen (or germinin, proposed by Voeller 1971) also overcomes the inhibition of spore germination in darkness (Voeller 1971; Schneller et al. 1990). However, except for Polypodium australe (Welling and Haufler 1993), and Aglaomorpha meyeniana (Naf 1966; 1969), Polypodiaceae s.s. has been thought neither to produce its own antheridiogen nor to respond to the antheridiogen of Pteridium aquilinum (L.) Kuhn, the most common antheridiogen known (Voeller 1971; Raghavan 1989). Genetic load is also an obstacle to successful intragametophytic selfing, and the degree of genetic load has been used to measure the sporophyte heterozygosity and probable breeding system (Lloyd 1974; Cousens 1988). However, most of the information known about the reproductive biology of ferns is from research on terrestrial ferns which encounter very different environmental events from epiphytic ferns.

*Polypodium pellucidum* Kaulf., a member of the Polypodiaceae and a diploid species with 2n = 74 (Manton 1951; Love et al. 1977), is endemic in Hawaii (Tryon and Tryon 1982; Lloyd 1974b). It grows on lava or is epiphytic in forests. Neither the gametophyte morphology nor the reproductive biology has been previously studied, except for a short note by Lloyd (1974b). Gametophytes of several epiphytic species of Polypodiaceae have been shown to be perennial and clone-forming (Chapters 1-5). This trait has been suggested to be an adaptation to the epiphytic habitat, enhancing the probability of gametophyte interaction in an environment (bryophyte mats) more challenging to intergametophytic mating than the terrestrial environment (Dassler 1995). Whether this trait is maintained in this species is
unknown. This paper investigates this issue in *Polypodium pellucidum*, addressing the following questions: (1) how are gametangia expressed in this species, (2) how much genetic load exists, (3) do gametophytes produce and/or respond to antheridiogen, (4) through which mating system are sporophytes produced, (5) how does gametophyte morphology relate to the reproductive system.

**Materials and methods**

Spores of *Polypodium pellucidum* were collected in August, 1992, from Hawaii (*Farrar 92-8-19-13*). Since antheridiogen of *Pteridium aquilinum* (L.) Kuhn (ApJ has been well studied (Naf et al. 1975), and gametophytes *Onoclea sensibilis* L. have been used to "safely identify antheridium-inducing activity" due to their complete lack of spontaneous antheridium production in young stages (Naf 1956), these two species were also used in this study to assay the activity of antheridiogen of *Polypodium pellucidum*. Spores of *Onoclea sensibilis* were collected from Marshall County, Iowa, in September, 1994 (*Chiou s.n.*), and those of *Pteridium aquilinum* were in part collected in June 1991 from Iowa City, Iowa (*Farrar 91-6-26-27*), and parts were kindly sent by Dr. David Wagner, collected from Eugene, Oregon, in August 3, 1994. Voucher specimens of *P. pellucidum* were deposited in the Ada Hayden Herbarium (ISC) of the Department of Botany in Iowa State University. Spores obtained from fertile fronds (*Chiou and Farrar 1994*) were stored in the refrigerator at about 5°C.

Spores were sown on 1% solidified agar media which contained Bold's macronutrients (Bold 1957) and Nitsch's micronutrients (Nitsch 1951), and a trace of ferric chloride (Peck 1985). Cultures were maintained under continuous, white fluorescent illumination of 2000-3000 lux. Temperature was maintained between 20-24°C.

Gametophyte morphology was observed under dissecting and compound microscopes. Pictures were either taken by auto-camera or hand-drawn using a drawing tube.
Sizes of gametophytes were measured as the width of the widest part. Gametophytes were observed every half month.

To harvest antheridiogen-enriched solution, the following procedure was used. One mg of spores of *P. pellucidum* and *P. aquilinum* was separately sterilized with 10% Clorox for 5 minutes and then rinsed with sterilized water. The sterilized spores were then suspended in 2 ml of sterilized water. Two drops of the spore suspension were placed into each of several petri dishes of 8.5 cm diameter containing about 50 ml of agar media. The cultures of *P. pellucidum* and *P. aquilinum* gametophytes were frozen at 4 months and 2 months old respectively, then thawed and the liquid extract collected. 2% and 10% dilutions of the extract were made by adding 20 drops (ca. 1 ml) of the extract into large petri dishes containing 50 ml of agar medium and the small petri dishes containing 10 ml of agar medium respectively. In addition to these extracts, GA3 (5x10^-5 g/ml) was also used to test antheridiogen response.

Considering the possibility that some antheridiogens might be short-lived (Emigh and Farrar 1977), that the time of harvesting extract might be not proper, or that the harvesting method may destroy antheridiogen activity, three additional experiments were conducted in which agar with mature growing gametophytes was used directly for the assay. In the first experiment, the agar with growing mature gametophytes was cut into 1 x 2 cm blocks. Then each agar block was turned on its side (turned block), so the gametophytes stood on one side of the agar block. Ten spores were sown on the top of each block and ten such blocks were put into each of two petri dishes. In the second experiment, agar supporting mature gametophytes was cut into four parts. Each part was placed upside down in another petri dish, and spores were sown on the upside-down agar (inverted block). In the third experiment, some gametophytes were removed from cultures to make a 1 cm wide lane (cleared block), and spores were sown on these cleared areas (between mature
gametophytes). All gametophyte cultures assayed for presence of antheridiogen were 4-month- and 2-month-old of *P. pellucidum* and *P. aquilinum* respectively.

Preparations of both extracts and blocks with growing gametophytes were used in tests of germination capacity in darkness and promotion of antheridium formation in the light. A complete randomized design was used. The percentage of plants forming antheridia was counted as the number of male plus bisexual individuals. Tests were repeated twice in the first experiment (Tables 5, 7) and four times in the second and third experiments (Tables 6, 8). At each observation time, 25 gametophytes were removed from each dish in the first experiment and 12 gametophytes were removed from each dish in the second and third experiments.

Capability of spore germination in darkness and the promotion of spore germination in the dark by antheridiogen were tested by wrapping two layers of aluminum foil around spore-sown petri dishes. These wrapped dishes were placed in a dark cabinet and unwrapped and examined one month later. Tests were repeated 2 (table 3) and 3 (table 4) times. Germination percentage was determined by counting 100 spores. The proportion of male plants was measured as the number of male gametophytes divided by the total number of dark-germinated gametophytes. A complete randomized design was used.

Genetic load was calculated by comparing isolated-spore and isolated-gametophyte cultures with paired-spore and paired-gametophyte cultures. These cultures were grown on "jelly-mold" plates, each with 20 cells. Each cell contained about 6 ml of agar medium. In each plate, a single spore was transferred onto each of 5 cells, and an one-month-old gametophyte which was still asexual was transferred onto each of another 5 cells. Another 10 cells were used as paired-spore and paired-gametophyte controls. Five such plates were separated by transparent plastic sheets and stacked into transparent plastic vegetable crispers for a total of 50 replicates of each culture type. The light intensity of these cultures were
maintained between 1500 lux (the bottom layer) to 3500 lux (the top layer). Plants were watered every two weeks after gametophytes were 4 months old. Sporophytes were determined to have been produced sexually by examination with a compound microscope. Genetic load was measured by counting the percentage of bisexual gametophytes failing to produce sporophytes. The five layers of plates were designated as blocks, and spore-culture vs. gametophyte-culture were designated as a split plot. Sporophytes were determined to have been produced sexually by examination with a compound microscope.

Results

Morphology

In multispore culture, spores began to germinate the second day after sowing, but germination was delayed for about one week if spores were stored in the refrigerator at 5°C for more than five months. Viability of spores remained at about 90% through 2.5-year of storage. After a basal cell, a rhizoid was usually the first cell produced on germination (Fig. 1). Rhizoids were brownish and non-septate. The basal cell underwent a second division (Fig. 3), resulting in a protonemal cell which subsequently produced a filament by serial divisions (Fig. 4). The lengths of filaments were various, usually four to six cells. In addition to the basal cell, rhizoids also emerged from other cells of the filament below the top cell. Sometimes two rhizoids appeared on the basal cell before the protonema cell developed (Fig. 2).

About one week after germination, a longitudinal division usually occurred at either the apical cell or the penultimate cell of the filament (Figs. 5, 6). Sometimes all the filament cells except the basal cell divided longitudinally (Fig. 6), or they might divide longitudinally more than once before the other dimensional divisions (Fig. 7). A large oil body always appeared in the basal cell during the young stages (Fig. 1-4). An obconical meristematic cell was built at the anterior margin by two oblique divisions (Figs. 5, 8, 9).
Figs. 1-18. Gametophyte development in *Polypodium pellucidum*. Figs. 1-14. Germination and early development. Figs. 15,16. Unicellular hairs on the marginal cells. Fig. 17. Unicellular hair on a surface cell. Fig. 18. Branched hairs. Bar = 0.1 mm.
The meristematic cell and its derivatives divided repeatedly, and the following expansion of these daughter cells eventually resulted in a broad spathulate thallus (Figs. 10, 11). A pluricellular meristem replaced the single meristematic cell later, and a nearly symmetrical cordate gametophyte formed eventually (Fig. 14). In some individuals, a lateral or sublateral meristem appeared (Figs. 12, 13).

Older gametophytes were usually with a notch meristem on the anterior part. However, in a crowded population, gametophytes were upright and slender and often spiraled. A midrib in the central region developed after the cordate gametophyte formed. As gametophytes grew older, their wings became lobed (Fig. 20).

In addition to the margin, rhizoids also occurred on both dorsal and ventral sides of the midrib as well as on the wings, but the latter were fewer than on the midrib or on the margin. Rhizoids emerging from archegonia were occasionally found (Fig. 24).

After attaining the later spathulate or early cordate stages, gametophytes produce hairs, most of which were unicellular, papillate, and secretory (Figs. 15-17, 38). They appeared on the margins and both dorsal and ventral sides of wings and midribs. Occasionally, branched hairs appeared (Fig. 18), usually on the older gametophytes. A secretion always capped the unicellular glandular hairs (Fig. 16). One gametophyte cell usually developed one hair but sometimes 2 hairs could be found on one cell (Fig. 38).

At about 2 months, antheridia appeared, usually on the posterior part (Figs. 19, 21), or sometimes near the apical meristem if a midrib had not formed, or on the margin (Fig. 22), especially on the irregular-shaped gametophytes (Fig. 23) which usually occurred at a later period or in high density patches of the culture. Archegonia also appeared at about 2 months, and were distributed on the midrib behind the meristem (Figs. 19-21). Both antheridia and archegonia were present on ventral and dorsal sides.

Vegetative propagation occurred on older gametophytes, arising from one or a few
Fig. 19-33. Mature gametophytes and response to experimental treatments. Figs. 19-24. Mature gametophytes of *Polypodium pellucidum*. Fig. 19. Unlobed hermaphroditic gametophyte. Fig. 20. Lobed female gametophyte. Fig. 21. Hermaphroditic gametophyte with 2 vegetative daughter gametophytes. Figs. 22-23. Male gametophytes. Fig. 24. Archegonium with a rhizoid. Figs. 25-33. Dark germination and antheridium formation in response to treatments. Figs. 25-31. *Polypodium pellucidum*. Fig. 25. Dark-growing gametophyte on an inverted block of *Polypodium pellucidum* gametophytes. Fig. 26. Dark-growing gametophyte on an inverted block of *Pteridium aquilinum* gametophytes. Fig. 27. Dark-growing gametophyte on agar medium containing 2% extract of *Polypodium pellucidum* Gametophyte cultures. Fig. 26. Dark-growing gametophyte on agar medium containing 10% extract of *Pteridium aquilinum* gametophyte cultures. Fig. 29. 1.5-month-old gametophyte growing on a cleared block of *Pteridium aquilinum* gametophytes. Fig. 30. 2.5-month-old gametophyte growing on medium containing 2% of the extract of mature *Polypodium pellucidum* gametophytes. Fig. 31. 1.5-month-old gametophyte growing on an inverted block of *Pteridium aquilinum* gametophytes. Figs. 32-33. *Onoclea sensibilis*. Fig. 32. 0.5-month-old gametophyte of *O. sensibilis* growing on medium containing 2% of extract of *Polypodium pellucidum* gametophyte cultures. Fig. 33. 1-month-old gametophyte of *O. sensibilis* growing on a cleared block of *Polypodium pellucidum* gametophytes. An = Antheridium. Ar = Archegonium. Bar = 0.1 mm, unless otherwise indicated.
Figs. 34-38. Antheridiogen experiments and gametophyte morphology. Figs. 34-37. Antheridiogen experiments. Fig. 34. Dark-growing gametophyte of *Polypodium pellucidum* on medium containing 2% extract of *P. pellucidum* gametophyte cultures. Fig. 35. A two-month-old gametophyte of *Polypodium pellucidum* on a side-turn-block of *P. aquilinum* gametophytes. Fig. 36. A 1.5-month-old gametophyte of *Polypodium pellucidum* on an inverted block of *P. aquilinum* gametophytes. Fig. 37. One-month-old *Onoclea sensibilis* growing on medium containing 2% extract of *P. pellucidum* gametophyte cultures. Fig. 38. Marginal unicellular hairs. Bar = 0.1 mm.
cells on the margin or on both surfaces (Fig. 21). A mat of overlapping branches formed later (Fig. 39). Gametophytes continued growing over two years and several sporophytes were derived from many of the individual gametophytes, which still remained growing after sporophyte production (Fig. 40).

**Sex sequence**

In multispore cultures, gametangia did not appear until 2 month after sowing spores. Sexual frequency varied with the culture time (Table 1). Archegonia developed earliest and female plants remained most common during the culture period of 4 months. Males appeared a little earlier than the hermaphrodites, and reached their highest proportion at 2.5 and 3 months and declined thereafter. These male gametophytes were relatively small and seemed to be developing from slow growing individuals, perhaps late-germinated spores. Hermaphrodites developed last, perhaps from either female or male gametophytes, and gradually became the principal type after 4 months (data not shown).

In isolated-cultures, sex expression percentages were not significantly different among different plate layers. On the other hand, except for males, sex expression differed significantly between the isospore- and isogametophyte cultures at 8-month after sowing spores (Table 2).

**Spore germination in darkness**

Spores of *P. pellucidum* did not germinate in the dark, or in darkness when GA3 was added, but did germinate in all other treatments, *i.e.*, when 2% and 10% extracts of *P. pellucidum* or *Pteridium aquilinum* gametophyte cultures were added to the media, and when spores were sown on the turned, inverted, or cleared agar blocks of mature gametophytes of *P. pellucidum* or *P. aquilinum* (Tables 3 and 4).

In general, the germination percentage of *P. pellucidum* in darkness was not significantly different between block and extract treatments in each of the two experiments.
Figs. 39-43. Gametophytes of *Polypodium pellucidum*. Fig. 39. One 6-month-old clonal gametophyte. Fig. 40. A single gametophyte clone with several sporophytes derived from it. Fig. 41. A 4 month old symgamous sporophyte (left) and an apogamous sporophyte (right). Fig. 42. A young apogamous sporophyte growing on the tip of the gametophyte. Fig. 43. Two apogamous "embryos" growing on the tips of proliferations. Bar = 0.1 mm for Figs. 42, 43; bar = 1 mm for Fig. 39; bar = 1 cm for Figs. 40, 41.
Table 1. The sexual frequency (%) and gametophyte size of *P. pellucidum* at different ages in multi-spore cultures.

<table>
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<th>Width (mm)</th>
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<tr>
<td></td>
<td>A M F H*</td>
<td>2.5 months</td>
<td>A M F H</td>
<td>3 months</td>
<td>A M F H</td>
<td>4 months</td>
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<td>25 23 42 10</td>
<td>12 21 48 19</td>
<td>4 12 41 43</td>
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* A = asexual, M = male, F = female, H = hermaphrodite.

Table 2. The sex expression (%) of *Polypodium pellucidum* in the isolated-spore (spore) and isolated-gametophyte (gametophyte) cultures at 8-months after sowing spores.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Male(^1)</th>
<th>Female(^2)</th>
<th>Bisexual(^3)</th>
</tr>
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<tbody>
<tr>
<td>Spore</td>
<td>7(a)</td>
<td>85(a)</td>
<td>8(b)</td>
</tr>
<tr>
<td>Gametophyte</td>
<td>4(a)</td>
<td>35(b)</td>
<td>61(a)</td>
</tr>
</tbody>
</table>

\(^{1,2,3}\) The same letter in parentheses means no significant difference in Duncan's multiple test (95\% c.l.). SEM = 4.30, 7.84, and 7.42 for \(^1\), \(^2\), and \(^3\) respectively.

(Tables 3, 4) except that the 2\% extract of *P. aquilinum* gametophyte cultures was significantly less effective than other *P. aquilinum* treatments (Table 3, conditions 5 vs. 6).

The promotion influence by *P. aquilinum* was higher than by *P. pellucidum* gametophytes for both of extracts and secretions of living gametophytes (Table 3, conditions 5, 6 vs. conditions 3, 4; Table 4, conditions 5, 6, 8 vs. conditions 3, 4, 7).

In dark cultures, antheridium onset by *P. pellucidum* gametophytes was promoted less by the 2\% extract of *P. pellucidum* gametophyte cultures than by the secretion from living gametophytes (Table 3, condition 3 vs. condition 4), but the influence of the 10\% extract was
Table 3. Average dark germination percentage and male percentage in *P. pellucidum* under different conditions (experiment I) at one month after sowing spores.

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Germination %**</th>
<th>Male %**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Light control</td>
<td>88 (a)</td>
<td>0 (c)</td>
</tr>
<tr>
<td>2. Dark control</td>
<td>0 (e)</td>
<td>0 (c)</td>
</tr>
<tr>
<td>3. Pp 2% extract</td>
<td>23 (d)</td>
<td>58 (b)</td>
</tr>
<tr>
<td>4. Pt turned block</td>
<td>24 (d)</td>
<td>93 (a)</td>
</tr>
<tr>
<td>5. Pt 2% extract</td>
<td>33 (c)</td>
<td>89 (a)</td>
</tr>
<tr>
<td>6. Pt turned block</td>
<td>72 (b)</td>
<td>88 (a)</td>
</tr>
</tbody>
</table>

* Pp = *P. pellucidum*, Pt = *P. aquilinum*.
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 3.51 and 8.58 for germination % and male % respectively, for conditions 3-6.

Table 4. Average dark germination percentage and male percentage in *P. pellucidum* under different conditions (experiment II) at one month after sowing spores.

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Germination %**</th>
<th>Male %**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Light control</td>
<td>87 (a)</td>
<td>0 (d)</td>
</tr>
<tr>
<td>2. Dark control</td>
<td>0 (c)</td>
<td>0 (d)</td>
</tr>
<tr>
<td>3. Pp inverted block</td>
<td>17 (b)</td>
<td>77 (bc)</td>
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<tr>
<td>4. Pp cleared block</td>
<td>18 (b)</td>
<td>82 (abc)</td>
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<tr>
<td>5. Pt inverted block</td>
<td>88 (a)</td>
<td>99 (a)</td>
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<tr>
<td>6. Pt cleared block</td>
<td>84 (a)</td>
<td>97 (a)</td>
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<tr>
<td>7. Pp 10% extract</td>
<td>17 (b)</td>
<td>71 (c)</td>
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<td>8. Pt 10% extract</td>
<td>81 (a)</td>
<td>94 (ab)</td>
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<tr>
<td>9. GA3 5x10^-5 g/ml</td>
<td>0 (c)</td>
<td>0 (d)</td>
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* Pp = *P. pellucidum*, Pt = *P. aquilinum*.
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 2.82, and 6.22 for germination % and male % respectively, for conditions 3-8.

not significantly different from secretions of living gametophytes (Table 4, condition 7 vs. conditions 3 & 4). On the other hand, all treatments by *P. aquilinum* were equally effective (Table 3, condition 5 vs. 6; Table 4, condition 8 vs. conditions 5 & 6). The influence of *P. aquilinum* was slightly higher than or not significantly different from that of *P. pellucidum* in promoting antheridium formation (Tables 3, 4).
Most dark-growing gametophytes produced antheridia at very young stages when they possessed only one or two prothallial cells (Figs. 25-27) which were pale-white and sometimes relatively elongated (Fig. 27), although some of these gametophytes remained asexual (Fig. 28). Many of the antheridia were mature and releasing active sperms (Fig. 34).

**Antheridium promotion in *Onoclea sensibilis***

Most of the gametophytes of *Onoclea sensibilis* generated antheridia by 0.5 months when the spores were sown on media contained the extract of *P. pellucidum*, or when spores were sown near the mature gametophytes of *P. pellucidum*, whereas all of them remained asexual under the control condition (Tables 5, 6). At one month and 1.5 months after spores were sown, a few of the gametophytes growing under the control condition produced antheridia but significantly less than those growing under treatment conditions (Tables 5, 6). The influence of the extracts (both 2% and 10%) was less than those of secretions of living gametophytes (Table 5, condition 2 vs. 3; Table 6, conditions 2 vs. 3 & 4). Antheridia were produced from very young filaments or on spathulate through heart-shaped prothalli. Usually only one or two antheridia were produced by young stages but antheridia will more abundant on the relatively mature gametophytes (Figs. 32, 33; Fig. 37) and active sperms were released.

**Antheridium promotion in *Polypodium pellucidum***

Gametophytes of *P. pellucidum* did not produce antheridia either under control condition or on the media containing GA3 at 1.5 months after the spores were sown, but did produce a few antheridia at two months and continuously generated them thereafter (Tables 7, 8). All other treatments significantly promoted antheridium formation at 1.5 months after sowing spores. The extract of either *P. pellucidum* or *P. aquilinum* promoted antheridium production less than the secretion of the living gametophytes at the first two months, but had the same effect at 2.5 months. Similarly, *P. aquilinum* had a slightly greater effect than *P.*
Table 5. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (c)**</td>
</tr>
<tr>
<td>2. Pp. 2% extract</td>
<td>78 (b)</td>
</tr>
<tr>
<td>3. Pp. turned block</td>
<td>94 (a)</td>
</tr>
</tbody>
</table>

* Pp = *P. pellucidum*.  
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 2.00, 5.66, and 2.83 for 0.5 months, 1 month, and 1.5 months respectively, for conditions 2-3.

Table 6. Average antheridium formation (%) by *Onoclea sensibilis* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (c)**</td>
</tr>
<tr>
<td>2. Pp 10% extract</td>
<td>75 (b)</td>
</tr>
<tr>
<td>3. Pp cleared block</td>
<td>81 (a)</td>
</tr>
<tr>
<td>4. Pp inverted block</td>
<td>86 (a)</td>
</tr>
</tbody>
</table>

* Pp = *P. pellucidum*.  
** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 2.46, 3.04, and 1.86 for 0.5 months, 1 month, and 1.5 months respectively, for conditions 2-4.

*P. pellucidum* did in earlier stage but the two had the same effect later (Tables 7, 8). Under the treatment conditions (except GA3 treatment), some of the gametophytes produced one to four antheridia on a one-celled prothallus stage (Figs. 31, 36). Most of them generated antheridia on filaments through the spathulate stage (Figs. 29, 30) and remained at these sizes. These antheridia released abundant active sperms (Figs. 35, 36).
Table 7. Average antheridium formation (%) by *Polypodium pellucidum* under different conditions (experiment I).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (e)**</td>
</tr>
<tr>
<td>2. Pp 2% extract</td>
<td>16 (d)</td>
</tr>
<tr>
<td>3. Pt 2% extract</td>
<td>64 (c)</td>
</tr>
<tr>
<td>4. Pp turned block</td>
<td>84 (b)</td>
</tr>
<tr>
<td>5. Pt turned block</td>
<td>100 (a)</td>
</tr>
</tbody>
</table>

* Pp = *P. pellucidum*, Pt = *P. aquilinum*.

** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 2.83, 2.83, and 2.00 for 1.5 months, 2 months, and 2.5 months respectively, for conditions 2-3.

Table 8. Average antheridium formation (%) by *Polypodium pellucidum* under different conditions (experiment II).

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5 months</td>
</tr>
<tr>
<td>1. Control</td>
<td>0 (e)**</td>
</tr>
<tr>
<td>2. GA3 5x10^-5 g/ml</td>
<td>0 (e)</td>
</tr>
<tr>
<td>3. Pp 10% extract</td>
<td>58 (d)</td>
</tr>
<tr>
<td>4. Pt 10% extract</td>
<td>65 (cd)</td>
</tr>
<tr>
<td>5. Pp cleared block</td>
<td>82 (b)</td>
</tr>
<tr>
<td>6. Pp inverted block</td>
<td>75 (bc)</td>
</tr>
<tr>
<td>7. Pt cleared block</td>
<td>98 (a)</td>
</tr>
<tr>
<td>8. Pt inverted block</td>
<td>98 (a)</td>
</tr>
</tbody>
</table>

* Pp = *P. pellucidum*, Pt = *P. aquilinum*.

** The same letter in parentheses in the same column indicates no significant difference in Duncan's multiple test (95% c.l.). SEM = 4.79, 5.62, and 5.51 for 1.5 months, 2 months, and 2.5 months respectively, for conditions 3-8.
Genetic load

As shown in table 9, gametophytes totally failed to produce sporophytes sexually in isolated-spore culture and nearly failed to do so in isolated-gametophyte cultures at 8 months after sowing spores. Thus the genetic load was very high, up to 98 in average, and there was no significant difference between the spore-culture and gametophyte-culture treatments. On the other hand, the paired-cultures were relatively successful in producing sporophytes (43% in average). More sporophytes were produced in the spore-cultures.

In fact, there was 21% of the isolated-cultures produced sporophytes. However, only 2% of them were produced through fertilization, whereas the others were generated through apogamy (Figs. 42, 43). None of these apogamous sporophytes grew as well as the sexually produced ones. They turned yellow-white, remained dwarfed (Fig. 41), and died eventually.

Table 9. The percentage of sporophyte production by syngamy (by surviving gametophytes) and genetic load (by bisexual gametophytes) by 8-month-old *P. pellucidum* on spore-culture (spore) and gametophyte-culture (gametophyte).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% sporophyte</th>
<th>load&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spore</td>
<td>gametophyte</td>
</tr>
<tr>
<td>isolated</td>
<td>0 (c)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2 (c)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>paired</td>
<td>63 (a)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>26 (b)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1, 2, 3 The same letter in parentheses in each items means no significant difference in Duncan's multiple test (95% c.l.). SEM = 6.12, 4.48, and 5.00 for 1, 2, and 3 respectively.

Discussion

Prothallic development in the genus *Polypodium* has been previously thought to be of Drynaria-type exclusively (Nayar 1962; Nayar and Raza 1969; Nayar and Kaur 1971). However, in this observation, based on the definition of Nayar and Kaur (1969; 1971),
Adiantum-type (Figs. 5, 9), Drynaria-type (Fig. 8), and Ceratopteris-type (Figs. 12, 13) have also been found, and thus the stability of the development type within the species is suspect. One possible reason is that every cell maintains the ability to divide. For the same reason, vegetative proliferations may occur at random over the whole gametophyte.

The mature gametophytes of *Polypodium* have been described as typically cordate (Momose 1967; Nayar 1962; Nayar and Raza 1969; Nayar and Kaur 1971). For *P. pellucidum*, besides being cordate in young and early mature stages, the older gametophytes became ruffled, lobed, and branched and lived for over two years although some of the older parts died. This clone-forming habit may function to increase the gametophytes' living space and to prolong the life span. These extended clonal and perennial gametophytes continuously form gametangia on their new proliferations, and thus enhance the possibility of interaction with other gametophytes established previously or later.

Intergametophytic mating has been recently thought to be the most common breeding system in homosporous ferns (Haufler and Soltis 1984; Soltis and Soltis 1990), and genetic load has been suggested to be the primary mechanism in promoting outcrossing (Masuyama 1979, 1986; Haufler et al. 1990; Haufler and Welling 1994). The high genetic load in this study suggests that sporophytes of *P. pellucidum* are mainly derived from intergametophytic mating. Lloyd (1974a) concluded that high levels of genetic load and intergametophytic mating characterized the species of the mature rain forest. *P. pellucidum* grows in mature forests as well as on near-forest lava areas where the heavy spore rain is possible, as without requiring sporophyte formation from isolated gametophytes (Ranker, per. comm.). Thus *P. pellucidum* can be a real pioneer species. Due to the male to hermaphroditic gametangium sequence on his soil culture, Lloyd (1974b) suggested that this species possessed maximum probabilities for intragametophytic mating, based on the hypothesis of Klekowski (1969). On the other hand, the sex sequence of female to hermaphroditic on the agar medium found in
both this culture and Lloyd's (1974b), has been proposed as facilitating either
intergametophytic mating (Klekowski 1969) or intragametophytic mating (Masuyama 1974a,
b). As reported by Lloyd (1974a), the correlation of the gametangium sequence and the
mating system may be too simplistic. In this case, genetic load seems to be more significant
than sexual sequence for determining the mating system.

Antheridiogen has been considered to be a mechanism to promote outcrossing
(Haufler and Welling 1994). The tests on both O. sensibilis and P. pellucidum gametophytes,
indicate antheridium formation is promoted significantly when the extract from P.
pellucidum gametophyte cultures is added to the media and when spores are sown near
mature P. pellucidum gametophytes (Tables 5-8). We conclude that antheridiogen (Apo)
exists in this species although it has not been purified and identified. Apo apparently is less
similar to GA3 (because the latter did not enhance antheridium formation) than it is to
antheridiogen of Pteridium aquilinum (Apt), to which gametophytes of P. pellucidum
responded with precocious antheridium formation.

The antheridiogen Apo perhaps was produced from gametophytes that were younger
than one month old, although male gametophytes in the multi-gametophyte cultures did not
occur until at 2 months old (Table 1). The gametophytes in the isolated-spore cultures were
mostly female (85%), whereas these in isolated-gametophyte cultures were mostly bisexual.
This is perhaps because these isolated-spore gametophytes had no opportunity to interact
with other gametophytes which might produce antheridiogen and induce antheridium onset
(Naf 1958). On the other hand, in the isolated-gametophyte cultures, the gametophytes were
transferred at one-month-old and 65% of them were with antheridia (i.e., male + bisexual)
when observed at 8 months old. This indicates that some of those gametophytes might have
been influenced by the antheridiogen secreted from other fast-growing gametophytes before
they were transferred (35% female may represent fast-growing and antheridium-insensitive
gametophytes). However, the possibility that transfer may injure the meristem and cause
decay the antheridium block also needed to be considered (Naf 1961).

Other indirect evidence of an antheridiogen system in *P. pellucidum* comes from the
pattern of male expression in multi-spore cultures, where male gametophytes were smaller
and antheridia were produced later than archegonia (Table 1). This fits the model of
antheridiogen function proposed by Naf (1963) who suggested that the fast-growing females
secrete antheridiogen which induces slow-growing gametophytes to produce antheridia.

Dopp (1950, cited from Naf 1963) found that antheridiogen extracts also retarded
gametophyte growth except at very low concentration. In this experiment, similarly, all
gametophytes growing in the treated cultures were smaller than those in untreated cultures
(data not shown). In treated cultures, gametophytes derived from spores sown near other
mature gametophytes (higher concentration of active substance) were smaller than those
growing on medium containing extract (lower concentration of active substance). We here
propose two possible reasons to explain the smaller gametophytes. One possibility is that the
potential vegetative growth is diverted to antheridium production as demonstrated by Naf
(1956). Although this can explain the stronger effect on small gametophytes growing near
other mature gametophytes, it does not explain why asexual gametophytes growing in treated
conditions are of sizes similar to male gametophytes. Another possibility is that an inhibitor
substance is secreted from the mature gametophytes as shown in some species of *Dryopteris*
(Bell 1958). To test this, we sowed spores of *P. pellucidum* in treatment conditions 5 and 6 in
the table 8, but using mature gametophytes of *Anemia phyllitis* instead of *P. pellucidum*. *A.*
*phyllitidis* is known to produce another group of antheridiogen (**A**<sub>N</sub>) which induces
antheridium production in *Anemia* but is inactive in families other than the Schizaeaceae. The
size of *P. pellucidum* in these cultures remained very small, similar in size to the precocious
male gametophytes described above, but all remained asexual during the 3-month culture
period (data not shown). This result indicates that inhibitors may be more universal and more closely related than antheridiogens among fern gametophytes. It seems that *P. pellucidum* can produce its own antheridiogen as well as inhibitor, and responds to both of them as well as to those produced by *Pteridium aquilinum*. On the other hand, for *A. phyllitidis*, *P. pellucidum* responds to its inhibitor but does not respond to the antheridiogen $A_{An}$. An alternative hypothesis is that antheridiogen functions in both antheridium promotion and gametophyte growth inhibition, but the efficiency of these two functions is different depending on the species. The statement that "antheridiogens actually affect size, and size influences sex expression" (Korpelainen 1994) is questioned here, at least in this species.

In polypodiaceae, neither antheridiogen presence nor response to $A_{pt}$ has been thought to exist (Voeller 1971; Raghavan 1989), except for a very weak response in *Aglamorpha meyeniana* (Naf 1966; 1969). However, recently Welling and Haufler (1993) demonstrated that antheridiogen also existed in *Polypodium australe*. Haufler et al. (1995) found that the frequency of outcrossing was consistent with random mating in the *Polypodium vulgare* complex, a species (complex) thought to be unaffected by antheridiogen (Voeller 1964; Welling and Haufler 1993), and further pointed that this contradicts the viewpoint "antheridiogen response and genetic load were coordinated in a predictable manner", stated by Schneller et al. (1990). In this study, the high level of genetic load and the existence of antheridiogen ($A_{po}$) in this species supports Schneller et al. (1990), and suggests that species of the *Polypodium vulgare* complex may be found to possess antheridiogen systems as well.

A substance secreted from mature gametophytes of *P. pellucidum* promotes spore germination in darkness, substituting for the requirement of light for spore germination (Tables 3, 4). We do not know if this substance is the antheridiogen $A_{po}$ or the so-called germinin (Voeller 1971). In general, functions of the substance for promoting antheridium
formation and substituting light requirement for spore germination are parallel (Voeller 1971), but Welling and Haufler (1993) hypothesized that these two substances may function independently. Our experiments show that antheridium formation (Tables 7, 8) and spore germination in darkness (Tables 3, 4) were similarly promoted by some chemical, but the effects had some degree of difference. Without further evidence, it is hard to determine whether germinin and antheridiogen are different or the same but with different required concentrations for promoting spore dark germination and for promoting antheridium production.

A soil spore bank has been demonstrated to exist for terrestrial species (Dyer and Lindsay 1992). For epiphytic species, fern spores are possibly deposited very deep in the bark or bryophyte mats where light intensity may be insufficient for spore germination. Antheridiogen (or germinin) may function in inducing germination of these "buried" spores. Through the antheridiogen influence, the resultant precocious gametophytes could produce antheridia and release active sperms to interact with older gametophytes. This model has been proposed previously (Voeller 1971; Schneller et al. 1990; Haufler and Welling 1994) for terrestrial ferns, and may fit epiphytic species as well.

In summary, Polypodium pellucidum, an endemic fern of Hawaii, produces gametophytes which are able to survive perennially through vegetative proliferations and produce antheridiogen which facilitates the establishment of male gametophytes. Antheridiogen (or possibly germinin) functions in inducing spore germinating in darkness. Sporophytes are produced primarily from outcrossing as evidenced by a high level of genetic load. The antheridiogen system (and germinin?), together with a clonal-forming habit and prolonged life span, provide a mechanism to promote interaction and outcrossing with other gametophytes which may develop from spores either dispersed later or previously buried in the lava or epiphyte substrate.
Acknowledgments

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Literature cited


8. THE MORPHOLOGY AND REPRODUCTIVE BIOLOGY IN *ELAPHOGLOSSUM SCHOTT*

This paper is prepared for publication in American Fern Journal

Wen-Liang Chiou¹, Donald R. Farrar¹, and Tom A. Ranker²

**Introduction**

The morphology of fern gametophytes has been studied and applied to taxonomy and systematics for a long time. Beyond the standard heart shapes depicted in most textbooks, great variations have also been shown (Atkinson 1973; Nayar and Kaur 1971). These variations include the ability to grow indeterminately and branch so that perennial gametophyte clones of considerable size may be produced (Farrar 1990; Dassler 1995). This morphological diversity may relate to the species' mating system and reproductive biology.

Four types of mating system in homosporous pteridophytes have been defined by Klekowski (1979): (1) intragametophytic selfing, (2) intergametophytic selfing, (3) intergametophytic crossing, and (4) intergametophytic mating. Among these mating systems, intragametophytic selfing has been previously suggested as being predominant in homosporous ferns due to the close proximity of antheridia and archegonia on bisexual gametophytes (Klekowski and Baker 1966; Klekowski 1973, 1979) and indeed there are some homosporous ferns which have very high rates of intragametophytic selfing (Crist and Farrar 1983; McCauley et al. 1985; Soltis and Soltis 1986a; Watano and Sahashi 1992). Polyploid species may also favor intragametophytic selfing due to duplicated alleles which mitigate the problem of recessive deleterious allele expression associated with selfing (Klekowski and Baker 1966; Lloyd 1974; Masuyama and Watano 1990). Recently, however, electrophoretic evidence has shown that intergametophytic mating is the most common...

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Several types of reproductive behavior can reduce the probability of intragametophytic selfing. Asynchronous maturation of male and female gametangia is one way to avoid intragametophytic selfing in bisexual gametophytes (Klekowski 1968, 1969; Masuyama 1974a, b). Possession of an antheridiogen system is another way to facilitate production of unisexual gametophytes, and thus promote intergametophytic mating (Naf et al. 1975; Voeller 1971; Hamilton and Lloyd 1991). Antheridiogen (or germinin, proposed by Voeller 1971) also overcomes the inhibition of spore germination in darkness (Voeller 1971; Schneller et al. 1990). However, except for Polypodium australe (Welling and Haufler 1993), and Aglaomorpha meyeniana (Naf 1966; 1969), Polypodiaceae s.s. has been thought neither to produce its own antheridiogen nor to respond to the antheridiogen of Pteridium aquilinum (L.) Kuhn, the most common antheridiogen known (Voeller 1971; Raghavan 1989). Genetic load is also an obstacle to successful intragametophytic selfing, and the degree of genetic load has been used to measure sporophyte heterozygosity and probable breeding system (Lloyd 1974; Cousens 1988). However, most of the information known about the reproductive biology of ferns is from research on terrestrial ferns which encounter very different environmental events from epiphytic ferns.

*Elaphoglossum*, a genus (Elaphoglossaceae) of tropical epiphytic ferns, occurs mostly in the New World and generally grows in mossy and foggy montane forests (Mickel 1985, Kuo 1990). The gametophyte morphology of some tropical American species (Stokey and Atkinson 1957) and a few Asian species (Momose 1967) has been studied, and reviewed by Atkinson (1973) and by Nayar and Kaur (1971). The reproductive biology of this genus, however, has not been investigated. Due to the epiphytic habitat and the nature of its bark and bryophyte substrate, dispersed spores are likely to be more separated, and the interaction of
the subsequent gametophytes more hindered than in terrestrial species (Dassler 1995). Thus gametophytes of the genus might be more likely to produce their sporophytes through intragametophytic selfing. In this study, several species of Elaphoglossum were investigated and the following events were addressed: morphology, gametangium expression, mating system, antheridiogen system, genetic load, and the possible significance of morphology related to the species' reproductive systems.

**Materials and methods**

Spores of *E. marginatum* (Wall ex Fee) Moore and *E. yoshinage* (Yatabe) Makino were collected in August, 1992 from Taiwan (*Chiou 14197 and 14200* respectively); spores of *E. alatum* Gaud. and *E. crassifolium* (Gaud.) Anderson & Crosby were collected in March, 1993, from Hawaii (*Farrar 93-3-26-1 and 93-3-20-1* respectively); *E. paleaceum* (Hook. & Grev.) Sledge was collected in August, 1992 from Hawaii (*Farrar 92-8-17-6*); *E. callifolium* (Bl.) Moore was kindly sent by Mr. S. J. Moore (*s.n.*) collected in December, 1993 from Taiwan. Since antheridiogen of *Pteridium aquilinum* (L.) Kuhn (A_p) has been well studied (Naf *et al.* 1975), and gametophytes of *Onoclea sensibilis* L. have been used to "safely identify antheridium-inducing activity" due to their complete lack of spontaneous antheridium production in early stages (Naf 1956), these two species were also used in this study to assay the activity of antheridiogen. Spores of *Onoclea sensibilis* were collected from Marshall County, Iowa, in September, 1994 (*Chiou s.n.*), and those of *Pteridium aquilinum* were in part collected in June, 1991 from Iowa City, Iowa (*Farrar 91-6-26-27*), and in part were kindly sent by Dr. David Wagner, collected from Eugene, Oregon, in August 3, 1994. Voucher specimens of *Elaphoglossum* were deposited in the Ada Hayden Herbarium (ISC) of the Department of Botany in Iowa State University. Spores obtained from fertile fronds (*Chiou and Farrar 1994*) were stored in the refrigerator at about 5°C.
Spores were sown on 1% agar-solidified media which contained Bold's macronutrients (Bold 1957) and Nitsch's micronutrients (Nitsch 1951), and a trace of ferric chloride (Peck 1985). Cultures were maintained under continuous, white fluorescent illumination of 2000-3000 lux. Temperature was maintained between 20-24°C.

Gametophyte morphology was observed under dissecting and compound microscopes. Pictures were either taken by auto-camera or hand-drawn using a drawing tube. Sizes of gametophytes were measured as the width of the widest part. Gametophytes were observed every half month.

To harvest antheridiogen-enriched solution, the following procedure was used. One mg of spores of *E. callifolium*, *E. crassifolium* and *P. aquilinum* were separately sterilized with 10% Clorox for 5 minutes and then rinsed with sterilized water. The sterilized spores were then suspended in 2 ml of sterilized water. Two drops of the spore suspension were placed into each of several petri dishes of 8.5 cm diameter containing about 50 ml of agar media. The cultures were frozen when *E. callifolium* and *E. crassifolium* were 10 months old and when cultures of *P. aquilinum* were 2 months old, then thawed and the liquid extract collected. 2% and 10% dilutions of the extract were made by adding 20 drops (ca. 1 ml) of the extract into large petri dishes containing 50 ml of agar medium and the small petri dishes containing 10 ml of agar medium respectively. In addition to these extracts, GA3 (5x10⁻⁵ g/ml) was also used to test antheridiogen response.

Considering the possibility that some antheridiogens might be short-lived (Emigh and Farrar 1977), that the time of harvesting extract might be not proper, or that the harvesting method may destroy antheridiogen activity, three additional experiments were conducted in which agar with mature growing gametophytes was used directly for the assay of response to antheridiogen. In the first experiment, the agar with growing mature gametophytes were cut into 1 x 2 cm blocks. Then the agar block was turned on its side (turned block), so the
gametophytes stood on one side of the agar block. Ten spores were sown on the top of each block and ten such blocks were put into each of two petri dishes. In the second experiment, agar supporting mature gametophytes was cut into four parts. Each part was placed upside down in another petri dish, and spores were sown on the upside-down agar (inverted block). In the third experiment, some gametophytes were removed from cultures to make a 1 cm wide lane (cleared block), and spores were sown on these cleared areas (between mature gametophytes). All gametophyte cultures assayed for presence of antheridiogen were 10 months and 2 months old for *Elaphoglossum* and *P. aquilinum* respectively.

The preparation of both extracts and blocks with growing gametophytes were used in tests of germination capacity in darkness and promotion of antheridium formation in the light. A complete randomized design was used. The percentage of plants forming antheridia was counted as the number of male plus bisexual individuals. Tests were repeated twice in the first experiment and in the treatment of 2% extract, and 25 gametophytes were removed from each dish at each observation time. In the second and third experiments, 10% extract and GA3 treatments, tests were repeated four times, and 12 gametophytes were removed from each dish at each observation time.

Capability of spore germination in darkness and the promotion spore germination in the dark by antheridiogen were tested by wrapping two layers of aluminum foil around spore-sown petri dishes. These wrapped dishes were placed in a dark cabinet, and unwrapped and examined one month later. Tests were repeated twice in each of 2% extract and turned block treatments, and 3 times in each of 10% extract, inverted block, and cleared block treatments. Germination percentage was determined by counting 100 spores. The proportion of male plants were measured as the number of male gametophytes divided by the total numbers of dark-germinated gametophytes. A complete randomized design was used.
Genetic load studies were performed only in *E. crassifolium* and *E. callifolium*, since bisexual gametophytes were only found in these two species, and was calculated by comparing isolated-spore and isolated-gametophyte cultures with paired-spore and paired-gametophyte cultures. These cultures were grown on "jelly-mold" plates, each with 20 cells. Each cell contained about 6 ml of agar medium. In each plate, a single spore was transferred onto each of 5 cells, and a one-month-old gametophyte which was still asexual was transferred onto each of another 5 cells. Another 10 cells were used as paired-spore and paired-gametophyte controls. Five such plates were separated by transparent plastic sheets and stacked into transparent plastic vegetable crispers for a total of 50 replicates of each culture type. The light intensity of these cultures were maintained between 1500 lux (the bottom layer) to 3500 lux (the top layer). Plants were watered every two weeks after gametophytes were 4 months old. Sporophytes were determined to have been produced sexually by examination with a compound microscope. Genetic load was measured by counting the percentage of bisexual gametophytes failing to produce sporophytes. The five layers of plates were designated as blocks, and spore-culture vs. gametophyte-culture were designated as a split plot.

For isozyme study, sporophytes of three species in Hawaii were collected by DRF in August, 1992 and May, 1993: *E. alatum*, *E. crassifolium*, and *E. paleaceum*. The grinding buffer and method followed Farrar (1990). Starch-gel electrophoresis and staining were conducted following Ranker *et al.* (1989). Six enzyme systems were scored in *E. alatum*, including aldolase (ALD), fructose-biphosphatase (FBP), HK, leucine aminopeptidase (LAP), malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PGD), and phosphoglucomutase (PGM). Nine enzyme systems were scored in *E. crassifolium*: ALD, esterase (EST), FBP, hexokinase (HK), LAP, MDH, 6PGD, shikimate dehydrogenase (SkDH), and triosephosphate isomerase (TPI). Ten enzyme systems were scored in *E.
paleaceum: ALD, FBP, HK, LAP, MDH, 6PGD, phosphoglucose isomerase (PGI), PGM, SkDH, and TPI.

For E. alatum and E. crassifolium, since they are diploid in Hawaii (Wagner, unpublished), when more than one isozyme appeared, the most anodal isozyme was assigned as "1" and the next most anodal "2", e.g., Tpi-1 vs. Tpi-2, (Gastony and Gottlieb 1985). To score the isozyme pattern of E. paleaceum, Werth's (1989) method was followed because it is tetraploid (Wagner, unpublished). The most anodal region of activity of each locus pair was given a numeric abbreviation of "1" (e.g., TPI-1). Members of the locus pair were abbreviated with letters. "a" designated to the more anodal region of activity (e.g., TPI-1a vs. TPI-1b). Isozyme data were calculated and analyzed by BIOSYS-1 (Release 1.7; Swofford and Selander 1989).

Results

Morphology

In multisporic cultures, spores began to germinate one week after sowing the spores. The maximum germination reached 95% for E. paleaceum, E. crassifolium and E. marginatum, 52% for E. alatum, 21% for E. callifolium and 16% for E. yoshinage at two weeks after sowing spores. Spores maintained about the same viability through 2-year storage except for E. alatum which failed to germinate after one-year storage.

A protonemal cell usually developed from the basal cell first (Figs. 1, 14), or sometimes the rhizoid appeared first, especially in E. alatum (Fig. 27), in which two rhizoids occasionally were found on the basal cell (Fig. 29). In most cases, a rhizoid did not derive from the basal cell until the filament or the spathulate stage had formed (Figs. 3, 5, 15-21, 36-38, 41), or even did not develop at all at young spathulate stages (Figs. 20, 21, 48). The basal cell and the protonemal cell subsequently produced a filament by serial divisions (Figs. 3, 4, 15-17, 29). The lengths of filaments varied from two to six cells. All E. paleaceum
Figs. 1-13. Gametophyte morphology of *E. crassifolium*. An = antheridium, Ar = archegonium, H = hair, M = meristem. Bar = 0.1 mm for Figs. 1-10; bar = 1 mm for Figs. 11-13.
Figs. 14-26. Gametophyte morphology of *E. callifolium* and *E. yoshinage*. Figs. 14-25. *E. callifolium*. Fig. 26. *E. yoshinage*. An = antheridium, Ar = archegonium. bar = 0.1 mm for Figs. 14-23, bar = 1 mm for Figs. 24-26.
Figs. 27-35. Morphology and development *E. alatum* gametophytes. Figs. 27-29. Early development. Fig. 30. A six month old gametophyte. Fig. 31. A forked rhizoid. Fig. 32. Hairs on marginal and superficial cells. Fig. 33. A 1.5 years old gametophyte. Fig. 34. A vegetative protrusion emanating from one cell. Fig. 35. A vegetative protrusion emanating from several cells and developing branches. Ar = archegonium. Bar = 0.1 mm, unless otherwise indicated.
Figs. 36-47. Gametophyte morphology and development of *E. marginatum*. Figs. 36-41. Early development. Figs. 42-43. Mature gametophytes. Figs. 44-47. Hairs. Ar = Archegonium, H = hair. Bar = 0.1 mm for Figs. 36-41; 44-47. Bar = 1 mm for Figs. 42, 43.
Figs. 48-54. Gametophyte morphology of some Elaphoglossum. Figs. 48, 49. *E. crassifolium*. Fig. 48. A spathulate prothallus without a rhizoid. Fig. 49. An 8 month old mat from a single spore. Figs. 50-52. *E. callifolium*. Fig. 50. A vegetative protrusion. Fig. 51. Maginal rhizoids and hairs, note one cell with both rhizoid and hair. Fig. 52. Short, dark brown rhizoids on the midrib. Figs. 53, 54. *E. alatum*. Fig. 53. Vegetative protrusions and hairs. Fig. 54. A one year old clone derived from a single spore. Bar = 1 cm for Fig. 49; bar = 1 mm for Fig. 54; bar = 0.1 mm for Figs. 48, 50-53.
gametophytes became brown and died after they attained the filament stage for unknown reasons.

About two weeks after germination, a longitudinal division occurred on one or several cells of the filament (Figs. 5, 19, 36, 39). Some filaments produced a branch which either soon stopped developing further or grew to small spathulate stages (Figs. 8, 18, 37). A wedge-shaped meristematic cell developed at the apex (Figs. 6, 7, 20, 38), or to the side if the apex terminated in a unicellular papillate hair (Figs. 21, 23). Sometimes an apical cell was not evident even in the late spathulate stage of prothallus development (Figs. 22, 40).

The meristematic cell and its derivative divided repeatedly, and the following expansion of these daughter cells resulted in a broad spathulate thallus (Figs. 6, 23). Hairs were derived from some marginal cells of the broad spathula (Figs. 6, 22, 23, 38, 40). A pluricellular meristem replaced the single meristematic cell later, and a nearly symmetrical cordate gametophyte formed eventually (Figs. 9, 41). The cordate gametophytes were almost as broad as long in some (Figs. 12, 43), but most of the others in this observation were relatively long and narrow (Figs. 24, 25, 42). The wings were usually ruffled and curled on the margin. A midrib at the central region developed after the cordate gametophyte formed. The midrib in some gametophytes was discontinuous (Figs. 25, 33). Both marginal and superficial unicellular hairs were abundant. Superficial hairs were located on both dorsal and ventral sides of the wing and midrib (Figs. 11-13, 24-26, 30, 33, 42, 43, 50, 53). Hairs were unicellular, papillate, and glandular and often capped by a secretion (Figs. 10, 32). In old gametophytes of *E. marginatum*, multicellular hairs appeared with abundant chloroplasts in the basal cells (Figs. 44-47).

Rhizoids growing from the margin of gametophytes were often clustered (Figs. 13, 25, 26, 33, 42, 43), but not always (Figs. 12, 24, 30). In addition to the margin, rhizoids also occurred on both dorsal and ventral sides of the midrib as well as on the wings, but those on
the latter were fewer than those on the midrib or on the margin. Usually, rhizoids in every species were similar regardless where they originated except in *E. callifolium* in which rhizoids on the midrib were relatively short and dark-brown (Fig. 52). Occasionally, a rhizoid and a hair emerged from the same prothallus cell (Fig. 51). Forked rhizoids were found infrequently (Fig. 31).

Before the midrib established, antheridia appeared on the posterior portion (Figs. 11, 12, 24, 25), except in *E. marginatum* and *E. alatum* (see sex sequence section below). Antheridia sometimes appeared on the margin of those gametophytes growing in crowded cultures. Some antheridia were mature and observed to open. Sperms were released but only "floated" (did not swim) to a short distance in all species except in *E. marginatum* in which no antheridia developed. Archegonia were distributed on the midrib (Figs. 12, 13, 24, 25, 33, 42, 43) of both ventral and dorsal sides. Antheridia and archegonia were positioned very closely (Figs. 24, 25) or even mixed together on some old gametophytes. In *E. yoshinage*, neither midribs nor archegonia were produced (see sex sequence section below).

As gametophytes grew older, sections of the margin became active and produced additional meristems (Figs. 11, 26). From these meristems, branches and additional meristems formed. Branches arising from one or a few cells also occurred (Figs. 33-35, 50, 53). Gametophytes continued growing over two years, and formed mats of overlapping branches (Figs. 13, 30, 33, 49, 54), especially in *E. alatum*, *E. crassifolium* and *E. yoshinage*. Relatively less branching occurred in *E. marginatum* and *E. callifolium* (Fig. 25). Some gametophytes remained unbranched when relatively old (Figs. 12, 24, 42, 43).

**Sex sequence**

In multisporic cultures, gametangium formation between 2 months old and 10 months old varied (Table 1) depending on the species. Antheridia developed earliest except for *E. marginatum* in which only archegonia appeared during the 2-year culture period.
Table 1. The sexual frequency (%) at different times in multi-spore cultures of 4 species of *Elaphoglossium*.

<table>
<thead>
<tr>
<th>Month</th>
<th><em>E. crassifolium</em></th>
<th><em>E. callifolium</em></th>
<th><em>E. marginatum</em></th>
<th><em>E. yoshinage</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>M</td>
<td>F</td>
<td>H*</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>88</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>52</td>
<td>48</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>16</td>
<td>84</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>

* A = asexual, M = male, F = female, H = hermaphrodite.  
** Only archegonia were found after 24 months.  
*** Only antherida were found after 24 months.

Hermaphrodites appeared later in *E. crassifolium* and *E. callifolium*, and all plants, without developing female only plants, became bisexual after 8 months old and 1 year old respectively, and sporophytes appeared thereafter. On the other hand, *E. marginatum* and *E. yoshinage* still remained unisexual at 2 years old (Table 1), and no sporophytes were produced by either. In *E. alatum*, archegonia did not appear until plants were one year old, and antheridia were not found at all, but some "floating" sperms were seen. Possibly antheridia were hidden among dense rhizoids. Although the gametophytes continued growth for the duration of the study, it became difficult to observe their gametangia at older ages, due to the impediment of thick masses of rhizoids. Very few sporophytes were produced after two years by gametophytes of *E. alatum*.

In isolated-cultures of *E. callifolium* and *E. crassifolium*, all gametophytes were bisexual after one year, regardless of which plate layers they grew in (data not shown), or whether they were initiated as spore or gametophyte cultures.

**Genetic load**

As shown in Table 2, all gametophytes of *E. crassifolium* and *E. callifolium* failed to produce sporophytes either in the isolated-spore or in the isolated-gametophyte cultures at 1
Table 2. The percentage of sporophyte production and genetic load of 1-year-old *E. crassifolium* and *E. callifolium* on spore-cultures (spore) and gametophyte-cultures (gametophyte).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% sporophyte</th>
<th>load</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spore gametophyte average</td>
<td>spore gametophyte average</td>
</tr>
<tr>
<td><em>E. callifolium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isolated</td>
<td>0 (b)¹</td>
<td>0 (b)¹</td>
</tr>
<tr>
<td>paired</td>
<td>24 (a)¹</td>
<td>18 (a)¹</td>
</tr>
<tr>
<td></td>
<td>0 (b)²</td>
<td>21 (a)²</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>E. crassifolium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isolated</td>
<td>0 (b)³</td>
<td>0 (b)³</td>
</tr>
<tr>
<td>paired</td>
<td>15 (a)³</td>
<td>19 (a)³</td>
</tr>
<tr>
<td></td>
<td>0 (b)⁴</td>
<td>17 (a)⁴</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

¹, ², ³, ⁴ The same letter means no significant difference in Duncan’s multiple test (95% c.l.).

year after sowing spores. Thus the genetic load was 100. On the other hand, the paired-cultures were relatively successful in producing syngamous sporophytes (17% and 21% average for *E. crassifolium* and *E. callifolium* respectively), and there was no significant difference between the spore-cultures and the gametophyte-cultures (data not shown).

Germination in darkness and antheridium promotion

Spores of *E. callifolium* and *E. crassifolium* did not germinate in darkness under any of the control or treatment conditions (data not shown).

Gametophytes of *Onoclea sensibilis* did not form more male individuals under treatment conditions than under control conditions in the 1.5-month culture period. Likewise no promotion of male gametophytes of *E. callifolium* and *E. crassifolium* occurred under treatments during the one-year observation (data not shown).

Isozyme analysis

In *E. alatum*, nine putative loci were resolved among six enzyme systems (Table 3). Allele frequencies are listed in table 3. A single allele was fixed in four loci (Fbp-1, Fbp-2, Lap, and Mdh-1). One of the other loci (6Pgd2) had four alleles whereas the remaining four
loci (Ald, Mdh-2, 6Pgd-1, and Pgm) had two alleles each (Table 3). The mean number of alleles per locus was 1.8; the percentage of polymorphic loci was 55.6; and the fixation index across polymorphic loci was 0.109 (Table 4). The significance of the fixation index was calculated by using Chi-square test of $H_0$ vs. $H_e$ at each polymorphic locus (Ranker et al. 1995), and the result indicates that the fixation index is not significantly greater than zero.

In *E. crassifolium*, 14 putative loci were resolved among nine enzyme systems (Table 5). A single allele was fixed in five loci (Ald, Est-1, Fbp-1, Fbp-2, and Tpi-2). Two of the other loci (Lap and Skd) had three alleles whereas the remaining seven loci (Est-2, Hk, Mdh-1, Mdh-2, 6Pgd1, 6Pgd2, and Tpi-1) had two alleles each (Table 5). The mean number of alleles per locus was 1.8; the percentage of polymorphic loci was 64.3; and the fixation index across polymorphic loci was 0.031 (Table 4). The significance of the fixation index was calculated (see above and Ranker et al. 1995), and the result indicates that the fixation index is not significantly greater than zero.

In *E. paleaceum*, seventeen putative duplicated locus pairs were scored among the ten enzyme systems (table 6). A single allele was fixed at each of nine locus pairs: Ald-a/b, Fbp-1a/b, Fbp-2a/b, Mdh-1a/b, Mdh-2a/b, Pgi-1a/b, Pgm-1a/b, Skd-2a/b, Tpi-2a/b. Two locus pairs (Hk-a/b, 6Pgd-1a/b) had fixed interlocus heterozygosity. Some sporophytes showed
Table 4. Genetic variability in 2 diploid species of *Elaphoglossum*.

<table>
<thead>
<tr>
<th>Species</th>
<th>$s^1$</th>
<th>$A^1$</th>
<th>$p^1$</th>
<th>Mean heterozygosity$^2$</th>
<th>Direct-count</th>
<th>H-W expected$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. alatum</em></td>
<td>28.8</td>
<td>1.8</td>
<td>55.6</td>
<td>0.088</td>
<td>0.108</td>
<td>0.109</td>
</tr>
<tr>
<td></td>
<td>(.7)$^4$</td>
<td>(.3)</td>
<td></td>
<td>(.049)</td>
<td>(.052)</td>
<td></td>
</tr>
<tr>
<td><em>E. crassifolium</em></td>
<td>34.5</td>
<td>1.8</td>
<td>64.3</td>
<td>0.076</td>
<td>0.086</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>(2.5)</td>
<td>(.2)</td>
<td></td>
<td>(.018)</td>
<td>(.024)</td>
<td></td>
</tr>
</tbody>
</table>

1 $s$ = mean sample size per locus, $A$ = mean number of alleles per locus, $P$ = % of polymorphic loci at 1% level, $F$ = fixation index across polymorphic loci.
2 Mean heterozygosity across all loci.
3 Unbiased estimate (Nei 1978).
4 Standard errors in parentheses.

Table 5. Allele frequency of *E. crassifolium*.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Sample</th>
<th>Allele number</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALD</td>
<td>41</td>
<td>1</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>1</td>
<td>1.000</td>
</tr>
<tr>
<td>EST-1</td>
<td>22</td>
<td>1</td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.932</td>
</tr>
<tr>
<td>EST-2</td>
<td>41</td>
<td>1</td>
<td>1.000</td>
</tr>
<tr>
<td>FBP-1</td>
<td>41</td>
<td>1</td>
<td>0.951</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.049</td>
<td></td>
</tr>
<tr>
<td>HK</td>
<td>41</td>
<td>1</td>
<td>0.828</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td>LAP</td>
<td>32</td>
<td>1</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td>MDH-1</td>
<td>41</td>
<td>1</td>
<td>0.951</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.049</td>
<td></td>
</tr>
</tbody>
</table>

variation in alleles at loci of Lap-a/b, 6Pgd-2a/b, Pgi-2a/b, Pgm-2a/b, Skd-1a/b, and Tpi-1a/b (Table 6). At each of these various loci, only a few individuals showed variation. Two genotypes were at each of these variable locus pairs except for Pgi-2a/b which had three genotypes. In total, eight genotypes were present among the sample sporophytes (Table 7).
Table 6. Allele frequencies in populations of *E. paleaceum*. Sample size is 35.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele</th>
<th>Frequency</th>
<th>Locus</th>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ald-a</td>
<td>1</td>
<td>1.00</td>
<td>pgI-1b</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Ald-b</td>
<td>1</td>
<td>1.00</td>
<td>PgI-2a</td>
<td>1</td>
<td>0.89</td>
</tr>
<tr>
<td>Fbp-1a</td>
<td>1</td>
<td>1.00</td>
<td>Fbp-1b</td>
<td>1</td>
<td>0.11</td>
</tr>
<tr>
<td>Fbp-1b</td>
<td>1</td>
<td>1.00</td>
<td>PgI-2b</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>Fbp-2a</td>
<td>1</td>
<td>1.00</td>
<td>Fbp-2b</td>
<td>1</td>
<td>0.99</td>
</tr>
<tr>
<td>Fbp-2b</td>
<td>1</td>
<td>1.00</td>
<td>PgM-1a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>HK-a</td>
<td>1</td>
<td>1.00</td>
<td>HK-b</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgm-1b</td>
<td>1</td>
<td>1.00</td>
<td>Lap-a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgm-2a</td>
<td>2</td>
<td>0.91</td>
<td>Lap-b</td>
<td>2</td>
<td>0.09</td>
</tr>
<tr>
<td>Pgm-2b</td>
<td>1</td>
<td>0.09</td>
<td>Mdh-1a</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Skd-1a</td>
<td>2</td>
<td>0.09</td>
<td>Skd-1b</td>
<td>2</td>
<td>0.97</td>
</tr>
<tr>
<td>Skd-2a</td>
<td>1</td>
<td>1.00</td>
<td>Skd-2b</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Skd-2b</td>
<td>1</td>
<td>1.00</td>
<td>Tpi-1a</td>
<td>1</td>
<td>0.94</td>
</tr>
<tr>
<td>Tpi-1b</td>
<td>2</td>
<td>0.03</td>
<td>Tpi-2a</td>
<td>2</td>
<td>0.06</td>
</tr>
<tr>
<td>Tpi-2b</td>
<td>2</td>
<td>0.97</td>
<td>Tpi-2b</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>Pgi-1a</td>
<td>1</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7. Description of multilocus genotypes of *P. paleaceum*.

<table>
<thead>
<tr>
<th>Lap</th>
<th>6Pgd-2a/b</th>
<th>Pgi-2a/b</th>
<th>Pgm-2a/b</th>
<th>Skd-1a/b</th>
<th>Tpi-1a/b</th>
<th>%</th>
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Discussion

Prothallus development in the genus *Elaphoglossum* has been described previously as Aspidium-type (Nayar and Kaur 1971). This study is in accordance with that description. The mature gametophytes of *Elaphoglossum* have been described as strape-shaped by Nayar and
Kaur (1971). Stokey and Atkinson (1957) depicted a broad type, a narrow type and an intermediate type depending on species. In this observation, we find that both broad and narrow (or sometimes intermediate) types appeared in every species. The older gametophytes become ruffled, lobed, and branched. Gametophyte clones formed by repeated branching. These clones in nature may function to increase the gametophytes' space and to prolong their life span. These extended clonal and perennial gametophytes continuously form gametangia on their new proliferations, and thus enhance the possibility of interaction with other gametophytes established previously or later. However, the capability of branching varies depending on species. For example, *E. crassifolium* and *E. alatum* quickly form many-branched clones, *E. callifolium* and *E. marginatum* slowly develop less-branched gametophytes, whereas *E. yoshinage* seems intermediate. Likewise, the probability of interaction with other gametophytes probably varies.

Intergametophytic mating has been recently considered to be the most common breeding system in homosporous ferns (Haufler and Soltis 1984; Soltis and Soltis 1990), and genetic load has been suggested to be the primary mechanism promoting outcrossing (Masuyama 1979, 1986; Haufler et al. 1990; Haufler and Welling 1994). The high genetic load (100%) found in this study suggests that sporophytes of *E. callifolium* and *E. crassifolium* are from highly outcrossing populations. This high level of genetic load and intergametophytic mating have been found to characterize species of mature rain forests (Lloyd 1974) where the species of *Elaphoglossum* grow. However, the male to hermaphroditic gametangium sequence has been suggested as facilitating intragametophytic mating (Klekowski 1969; Masuyama 1974a, b). This seeming contradiction indicates that the mating system cannot be determined by gametangium expression only (Lloyd 1974). Because all gametophytes in paired-cultures originated from the same sporophyte, the low
production of sporophytes in paired cultures may indicate a very high number of recessive lethals.

Antheridiogen has been considered to be a mechanism promoting outcrossing (Peck et al. 1990; Haufler and Welling 1994). However, we did not find promotion of antheridium production in *Onoclea sensibilis* or in the two species of *Elaphoglossum* when the extract of *Elaphoglossum* cultures was added to the media or when spores were sown near mature *Elaphoglossum* gametophytes. The antheridium promotion also did not occur when GA3 or antheridiogen of *Pteridium aquilinum* was applied to *Elaphoglossum* cultures. Sexual expression in multispore cultures also does not fit the mode of antheridiogen function proposed by Naf (1963). In addition, all spores tested under darkness did not germinate either in control or in treatment conditions. These results indicate no antheridiogen production or response in the tested species. Here intergametophytic mating (indicated by the genetic load experiment) without an antheridiogen system also contradicts the hypothesized correlation between the genetic load and antheridiogen response (Schneller et al. 1990). In *Elaphoglossum*, outcrossing may be promoted solely by genetic load and the gametophyte's clone-forming growth habit.

The fixation index values, calculated from isozyme data, for *E. alatum* and *E. crassifolium* are not significantly different from zero and suggest that these samples of two species are in Hardy-Weinberg random mating and derived from random mating populations. The breeding system of *E. crassifolium* suggested by isozyme patterns is accordance with results of isolated-cultures which showed a very high genetic load and indicated an intergametophytic mating system in this species. However, absence of an antheridiogen system is contrary to the demonstrated correlation between antheridiogen and high genetic load (Schneller et al. 1990).
The mating system of diploid species can be derived from isozyme electrophoretic patterns. However, electrophoretic patterns for *E. paleaceum* revealed fixed interlocus heterozygosity at locus-pairs Hk-a/b and 6Pgd-1a/b, indicating that these samples from Hawaii are polyploid, probably allopolyploid. Because of this, accurate counts of heterozygous individuals and estimates of outcrossing are not possible, but at least one outcrossed individual (Pgi-2a\(^{11}/b^{12}\)) was among the 35 samples in Hawaii.

Assuming allopolyploidy and no mutation, the maximum number of genotypes per locus-pair indicates the minimum number of hybridization events originating the species (Ranker *et al.* 1994). Three genotypes at the locus-pair Pgi-2a/b suggest at least three hybridization events in these samples. On the other hand, the total of eight multilocus genotypes indicates that the number of independent hybridization events may be as high as eight (Ranker *et al.* 1994). Each multilocus genotype possibly represent a distinct hybridization event, assuming neither sexual recombination nor mutation has played a major role in generation of the genotypes in these samples. On the other hand, the very low frequency of all the alternative alleles makes mutation a plausible explanation for their origin.

The observation of released sperm floating but not swimming is puzzling. We do not know whether this is due to inappropriate culture conditions or whether this is an expression of gametangial disfunction. It seems unlikely that archegonia could be found and penetrated by non-swimming sperm, even in foggy rainforests. We also wonder why *E. marginatum* and *E. yoshinage* maintained female and male respectively during the 2-year growth period, and by what breeding system they produce sporophytes which are not rare in their natural habitats. Another interesting observation is the relationship between midrib and archegonia. Neither midribs nor archegonia were produced by gametophytes of *E. yoshinage* suggesting the former may be necessary for the latter. Field research and more laboratory work are necessary to understand their reproductive biology.
In summary, due to the epiphytic habitat, dispersed spores of *Elaphoglossum* are likely to be functionally isolated than are spores of terrestrial species. This would seem to promote intragametophytic selfing. The isozyme data of *E. alatum* and *E. crassifolium* suggest that their sporophytes are derived from highly outbreeding populations. The high genetic load of *E. callifolium* and *E. crassifolium* indicates low probabilities of successful intragametophytic mating. It was not possible to determine the breeding system of the polyploid species *E. paleaceum* from isozyme data and gametophyte cultures did not survive. To produce a sporophyte through intergametophytic mating, a gametophyte has to interact with other gametophyte(s). The clone-forming habit and perennial growth of *Elaphoglossum* gametophytes may function to prolong the life span and to enlarge the space occupied by individual gametophyte, and thus to promote interaction and outcrossing with other gametophytes. The two tested species, *E. callifolium* and *E. crassifolium*, lack their own antheridiogen and are insensitive to that of *Pteridium aquilinum*. Intergametophytic mating may be maintained by genetic load and promoted by perennial and clone-forming growth habit.

**Acknowledgments**

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**Literature cited**


GENERAL CONCLUSION

Introduction

The fern gametophyte, which can be male, female, or hermaphroditic, carries antheridia and archegonia which produce sperm and eggs, respectively. It is the site of fertilization, subsequent sporophyte production, and the generation of genetic variation through different reproductive systems. Klekowski (1979) defined four types of mating systems in homosporous ferns: intragametophytic selfing, intergametophytic selfing, intergametophytic crossing, and intergametophytic mating. Due to the high frequency of relatively high basal chromosome numbers in homosporous ferns, Klekowski and Baker (1966) reported that polyploidy was common in ferns. From these observations, Klekowski (1973) proposed that 1) most ferns reproduce sexually through self-fertilization by single bisexual gametophyte, 2) the enhanced heterozygosity provided by the duplicated genomes of polyploids protects against expression of lethal genes through intragametophytic selfing, and 3) homoeologous chromosome pairings between duplicated sets could serve to release genetic variation (Klekowski 1973). Diploid ferns tend to reproduce by intergametophytic mating whereas tetraploid ferns most commonly reproduce by intragametophytic selfing (Masuyama 1979, Masuyama et al. 1987, Masuyama and Watano 1990).

On the other hand, isozyme electrophoretic studies have recently revealed that functional diploidy, with no evidence of duplicated genomes, and intergametophytic mating is predominant among terrestrial ferns with basal chromosome number (Haufler and Soltis 1984; Haufler 1987; P. Soltis and D. Soltis 1989; P. Soltis and D. Soltis 1990; D. Soltis and P. Soltis 1992). High genetic load, which causes genetic inbreeding depression (inviability or reduced vitality of sporophytes produced through selfing), has been suggested to be the primary mechanism promoting outcrossing (Haufler et al. 1990; Haufler and Welling 1994). Other indirect evidence for intergametophytic mating has been indicated by antheridiogen
research (Voeller 1971; Naf 1979). Antheridiogen, which is secreted from older, fast-growing gametophytes, induces younger, slow-growing gametophytes to produce antheridia and results in the simultaneous occurrence of male and female gametophytes, thus increasing the probability of outcrossing. A correlation between antheridiogen response and frequency of outcrossing has been demonstrated in laboratory research (Schneller et al. 1990). An additional function of antheridiogen, [or possibly another chemical called germinin (Voeller 1971)], is the induction of spore germination in darkness, e.g., buried spores. Gametophytes from such spores become potential male gametophytes and further enhance the probability of outcrossing.

A few studies have indicated that some epiphytic ferns are also outbreeders (Ranker 1992; Masuyama et al. 1987; Haufler et al. 1995). However, it seems probable that epiphytic species face a more difficult challenge in producing interactive populations of gametophytes, particularly when spore dispersal is more than a few meters. Most epiphytic fern habitats, especially in tropical and subtropical rainforests, are covered by mats of bryophytes. These mats likely create unfavorable conditions for fern spore germination (because of periodic desiccation near the surface of the mat and by the darkness deep within the mat where desiccation is less of a problem), for gametophyte growth (because of competition and physical obstruction by the surrounding bryophytes), and for sperm transfer (because of the lack of a direct route between plants suspended in the bryophyte mat) (Dassler 1995). Clone formation and perennial growth habit of gametophytes have been recently suggested as being an adaption to decrease interaction distance and to promote outcrossing in epiphytic ferns (Dassler 1995). This paper reviews and summerized previous studies about gametophyte morphologies, growth habits, antheridiogen systems, genetic load and reproductive behavior of some epiphytic ferns in the Polypodiaceae and *Elaphoglossum* (Elaphoglossaceae).
Materials and methods

The following species have been used in this study:

Polypodiaceae

*Campyloneurum angustifolium* (Swartz) Fee,
*C. phyllitidis* (L.) Presl,
*Lepisorus thunbergianus* (kaulf.) Ching,
*Microgramma heterophylla* (L.) Wherry,
*Phlebodium aureum* (L.) J. Smith,
*Phymatosorus scolopendria* (N. L. Burm.) Pichi Serm.,
*Polypodium pellucidum* Kaulf.,

Elaphoglossaceae

*Elaphoglossum alatum* Gaud.,
*E. callifolium* (Bl.) Moore,
*E. crassifolium* (Gaud.) Anderson & Crosby,
*E. marginatum* (Wall ex Fee) Moore,
*E. paleaceum* (Hook. & Grev.) Sledge,
*E. yoshinage* (Yatabe) Makino.

In multispore cultures, spores were sown on 1% agar-solidified media which contained Bold's macronutrients (Bold 1957), Nitsch's micronutrients (Nitsch 1951), and a trace of ferric chloride (Peck 1985). Cultures were maintained under continuous, white fluorescent illumination of 2000-3000 lux. Temperature was maintained at between 20-24°C.

To harvest antheridiogen-enriched solutions, agar supporting mature gametophytes was frozen then thawed and the liquid extract was collected. This method has been widely used (Schedlbauer and Klekowski 1972; Nester and Schedlbauer 1982). In addition to these extracts, GA3 (5x10^{-5} g/ml) was also used to test for antheridiogen response since response
to this hormone has been found in some fern families (Voeller and Weinberg 1969; Emigh and Farrar 1977).

Considering the possibility that some antheridiogens might be short-lived (Emigh and Farrar 1977), that the timing of harvesting extract might be not appropriate, or that the harvesting method may destroy antheridiogen activity, three additional experiments were conducted in which agar with mature growing gametophytes was used directly for the assay. In the first experiment, the agar with growing mature gametophytes was cut into 1 x 2 cm blocks. Then each agar block was turned on its side (turned block), so that the gametophytes stood on one side of the agar block. Spores were sown on the top of each block (Fig. 1). In the second experiment, agar supporting mature gametophytes was cut and placed upside down in another petri dish, and spores were sown on the upside-down agar (inverted block, Fig. 2). This method has been used previously but with removal of the mature gametophytes (Dubey and Roy 1985). In the third experiment, some of the gametophytes were removed from cultures to make a one cm wide lane (cleared block), and spores were sown on these cleared areas (Fig. 3). Since antheridiogen of *Pteridium aquilinum* (Apt) has been well studied (Naf et al. 1975), and gametophytes of *Onoclea sensibilis* L. have been used to "safely identify antheridium-inducing activity" due to their complete lack of spontaneous antheridium production in young stages (Naf 1956), these two species were also used in this study to assay the activity of antheridiogen of other species.

The preparation of both extracts and blocks with growing gametophytes were used in tests of promotion of antheridium formation in the light and germination capacity in darkness. Spore germination capacity in darkness and the promotion of spore germination in the dark by antheridiogen were tested on media treated as in experiments for antheridium promotion and then wrapped with two layers of aluminum foil around the spore-sown petri
Figs. 1-6. Gametophyte cultures and experimental response. Figs. 1-5.
Gametophyte cultures. Fig. 1. Turned blocks growing mature gametophytes. Fig. 2. An inverted block. Fig. 3. A cleared block. Fig. 4. Aluminum-foil-wrapped petri dishes for experiment on spore germination in darkness. Fig. 5. Isolated and paired cultures for genetic load experiments. Fig. 6. 1.5 month old gametophytes of *Onoclea sensibilis* growing on media containing (right) and without (left) culture extract of *P. aquilinum* gametophytes.
These wrapped dishes were placed in a dark cabinet, then unwrapped and examined one month later.

Genetic load was calculated by comparing isolated-spore and isolated-gametophyte cultures with paired-spore and paired-gametophyte cultures. These cultures were grown on "jelly-mold" plates. Single spores and single one-month-old gametophytes which were still asexual, and paired spores and paired one-month-old gametophytes were transferred into five cells of each plate respectively. Five such plates were separated by transparent plastic sheets and stacked into transparent plastic vegetable crispers (Fig. 5). The light intensity was maintained between 1500 lux (the bottom layer) to 3500 lux (the top layer). Plants were watered every two weeks after gametophytes were 4 months old. Sporophytes were determined to have been produced sexually by examination with a compound microscope. Genetic load was measured by counting the percentage of bisexual gametophytes failing to produce sporophytes.

For isozyme observation, the grinding method and buffer followed Farrar (1990). Starch-gel electrophoresis and staining were conducted following Ranker et al. (1989). Isozyme data were calculated and analyzed by BIOSYS-1 (Release 1.7; Swofford and Selander 1989). The ploidy level of sporophytes tested was determined by isozyme patterns and by previous chromosome counts.

Results and discussion

Morphology and growth habits

In Elaphoglossum, development of all species studied followed the Aspidium pattern of Nayar and Kaur (1971), whereas in the Polypodiaceae, development patterns varied depending on the species. The Drynaria-type appeared in every polypodiaceous species. An additional type (Ceratopteris-type) occurred in Campyloneurum phyllitidis, Phlebodium
aureum, and Polypodium pellucidum. In Polypodium pellucidum, the Adiantum-type, and in Microgramma heterophylla, the Aspidium-type also appeared (Table 1).

A cordate-thalloid morphology appeared in adult gametophytes of every species studied. In addition, strap-like gametophytes developed in the genus Elaphoglossum, and three species of Polypodiaceae: Campyloneurum phyllitidis, Lepisorus thunbergianus, and Microgramma heterophylla.

Considering habitat, growth form, and morphology, Dassler (1995) classified gametophytes into three types: 1) short-lived and cordate-thalloid, 2) persistent, strap-like or ribbon-like, and clone-forming, 3) persistent, strap-like, ribbon-like or filamentous, clone-forming, and gemmae-producing. She concluded that Polypodiaceae and Elaphoglossum belong to the second category. In our observation, seven species of Polypodiaceae and five species of Elaphoglossum were all long-lived and clone-forming (Table 1). However, some species maintained a cordate shape in both the original gametophyte and proliferations unless they grew in very high densities or under very low light intensity where they became slender and strap-like.

Clones are derived from two types of branching: 1) a segment of marginal cells becomes a pleuricellular meristem which produces a branch, 2) one or a few marginal cells produce a proliferation which elongates and expands to some degree, then develops a pleuricellular meristem and branch. The second type is common to gametophytes of all species studied whereas the first type is less frequent (Table 1). Several branches could develop on one gametophyte in both types.

**Antheridium promotion by antheridiogen**

Antheridium production by Elaphoglossum callifolium and E. crassifolium gametophytes was promoted neither by other mature gametophytes of the same species nor by gametophytes of Pteridium aquilinum (Table 2). In addition, both culture extracts and
Table 1. Summary of morphology and growth habits in some epiphytic fern gametophytes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Habitat</th>
<th>Development type</th>
<th>Mature form</th>
<th>Branch type</th>
<th>Longevity</th>
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<td>D</td>
<td>C, CL</td>
<td>1, 2</td>
<td>Long-lived</td>
</tr>
<tr>
<td>C. phyllitidis</td>
<td>E, R</td>
<td>C, D</td>
<td>C, S, CL</td>
<td>1, 2</td>
<td>Long-lived</td>
</tr>
<tr>
<td>Lepisorus thunbergianus</td>
<td>E, R</td>
<td>D</td>
<td>C, S, CL</td>
<td>1, 2</td>
<td>Long-lived</td>
</tr>
<tr>
<td>Microgramma heterophylla</td>
<td>E, R</td>
<td>Ad, C, D</td>
<td>C, S, CL</td>
<td>1, 2</td>
<td>Long-lived</td>
</tr>
<tr>
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<td>C, S, CL</td>
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<td>Long-lived</td>
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<td>As</td>
<td>C, S, CL</td>
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<td>C, S, CL</td>
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<tr>
<td>E. yoshinage</td>
<td>E, R</td>
<td>As</td>
<td>C, S, CL</td>
<td>1, 2</td>
<td>Long-lived</td>
</tr>
</tbody>
</table>

1 E = epiphytic, H = hemiepiphytic, R = rupestral, T = terrestrial.
2 Ad = Adiantum-type, As = Aspidium-type, C = Ceratopteris-type, D = Drynaria-type, based on Nayar and Kaur (1971).
4 Branch type is defined in the text.
Table 2. Antheridium production (%) by gametophytes under different conditions at 2.5 months old (+) or 3 months old.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Species</th>
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<th>Lt+</th>
<th>Mh+</th>
<th>Pa</th>
<th>Pp+</th>
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<td>Sp turned</td>
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<tr>
<td>Sp cleared</td>
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<td>***</td>
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<tr>
<td>Sp inverted</td>
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<td>Pt 2% extract</td>
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<td>Pt 10% extract</td>
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</tbody>
</table>

1 Ca = Campyloneurum angustifolium, Cp = Campyloneurum phyllitidis, Lt = Lepisorus thunbergianus, Mh = Microgramma heterophylla, Pa = Phlebodium aureum, Pp = Polypodium pellucidum, Ps = Phymatosorus scolopendria, Eca = Elaphoglossum callifolium, Ecr = Elaphoglossum crassifolium.

2 For treatments see "materials and methods". "Sp" means that species producing treatments are the same species as the one tested, "Pt" means that gametophytes producing treatments are Pteridium aquilinum. [GA3] = 5x10^-5 g/ml.

3 "-" represents no response. "**", "***", "****" represents antheridium production significantly greater than that of control treatments, alpha = 0.05, 0.01, & 0.001 respectively.
living gametophytes of *E. callifolium* and *E. crassifolium* did not promote antheridium formation on gametophytes of *Onoclea sensibilis* (Table 3) which is the species most sensitive to antheridiogen of *P. aquilinum*. Sexuality expression in multispore cultures did not fit Naf's (1963) model of antheridiogen operation either. These results suggest that the two species of *Elaphoglossum* tested neither produce their own antheridiogen nor responded to antheridiogen of *P. aquilinum*. If so, the observation of the correlation between antheridiogen and genetic load (Schneller *et al.* 1990) is not true for *E. callifolium* and *E. crassifolium* (see genetic load section below).

On the other hand, antheridium formation in all seven species of Polypodiaceae was promoted by culture extracts (except *Phlebodium aureum*), and by secretions of mature living gametophytes of the same species and of *P. aquilinum* (Table 2). Furthermore, secretion by gametophytes of all the Polypodiaceae species significantly promoted antheridium formation by gametophytes of *Onoclea sensibilis* (Table 3). These results suggest that these species produce and respond to their own antheridiogen and respond to that of *P. aquilinum*. However, antheridiogen production and the antheridiogen concentration required to promote antheridium formation varies among species. Some of these species did not respond to culture extracts of the same species or to culture extracts of *P. aquilinum* gametophytes although they did respond to nearby mature, growing gametophytes. This suggests that the antheridiogen concentration required to promote antheridium formation is relatively high, which is one of the possible reasons that most Polypodiaceae species have been considered to not possess an antheridiogen system or to not respond to antheridiogen *A*_pt (Voeller 1971; Raghavan 1989), with a few exceptions (Naf 1966, 1969; Welling and Haufler 1993). Other possible explanations of non-promotion of antheridium formation by extracts is that the extraction method might destroy antheridiogens or that the antheridiogen may retain activity for only short time (Emigh and Farrar 1977). However, the fact that antheridium formation
Table 3. Antheridium production (%) by 1.5-month-old gametophytes of *Onoclea sensibilis* under different conditions.

<table>
<thead>
<tr>
<th>Treatments 1, 2</th>
<th>2% extract</th>
<th>10% extract</th>
<th>Turned block</th>
<th>Inverted block</th>
<th>Cleared block</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>*3</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Cp</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Lt</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Mh</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Pa</td>
<td>-</td>
<td>-</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Pp</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Ps</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Eca</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ecr</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2 Treatments see "materials and methods".
3 "-" indicates no response. "+", "***", "****" indicates antheridium production significantly greater than that of control treatments, alpha = 0.05, 0.01, & 0.001 respectively.

by *O. sensibilis* was significantly promoted by culture extracts of all species tested, except *P. aureum*, indicates that these antheridiogens are stable. That GA3 did not promote antheridium formation in any species tested indicates that these antheridiogens may not be closely related to GA3.

**Dark germination promoted by antheridiogen or germinin**

None of the spores of *Elaphoglossum* tested germinated in darkness under any treatments (Table 4). On the other hand, all species of Polypodiaceae tested except
Table 4. Dark germination of spores under different conditions at one month after spores were sown.

<table>
<thead>
<tr>
<th>Treatments²</th>
<th>Species¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca</td>
</tr>
<tr>
<td>Sp 2% extract</td>
<td>- 3</td>
</tr>
<tr>
<td>Sp 10% extract</td>
<td>-</td>
</tr>
<tr>
<td>Sp turned</td>
<td>-</td>
</tr>
<tr>
<td>Sp cleared</td>
<td>+</td>
</tr>
<tr>
<td>Sp inverted</td>
<td>+</td>
</tr>
<tr>
<td>Pt 2% extract</td>
<td>-</td>
</tr>
<tr>
<td>Pt 10% extract</td>
<td>+</td>
</tr>
<tr>
<td>Pt turned</td>
<td>-</td>
</tr>
<tr>
<td>Pt cleared</td>
<td>*</td>
</tr>
<tr>
<td>Pt inverted</td>
<td>+</td>
</tr>
<tr>
<td>GA3</td>
<td>-</td>
</tr>
</tbody>
</table>

¹ Ca = Campyloneurum angustifolium, Cp = Campyloneurum phyllitidis, Lt = Lepisorus thunbergianus, Mh = Microgramma heterophylla, Pa = Phlebodium aureum, Pp = Polypodium pellucidum, Ps = Phymatosorus scolopendria, Eca = Elaphoglossum callifolium, Ecr = Elaphoglossum crassifolium.

² For treatments see "materials and methods". "Sp" means that species producing treatments are the same as the species tested, "Pt" means that gametophytes producing treatments are Pteridium aquilinum. [GA3] = 5x10⁻⁵ g/ml.

³ "-" represents no germination. "+" represents germination but % is not significantly greater than zero. "**, ***", and "****" represents germination significantly greater than zero, alpha = 0.05, 0.01, & 0.001 respectively.
Phlebodium aureum germinated in darkness under one or more treatments (Table 4). Apparently, a substance secreted from mature gametophytes of the Polypodiaceae species tested and from gametophytes of *P. aquilinum* substitutes for the light requirement for spore germination. Traditionally, this substance has been considered to be antheridiogen although Voeller (1971) proposed a chemical different from antheridiogen which he called germinin. In general, functions of the substance in promoting antheridium formation and the substance inducing spore germination in darkness are parallel (Voeller 1971), but Welling and Haufler (1993) hypothesized that these two substances might function independently.

In these studies, except for *P. aureum*, secretions of Polypodiaceae gametophytes promoted both spore germination in darkness and antheridium formation in the same species, whereas culture extracts of Polypodiaceae gametophytes promote both responses (*Polypodium pellucidum*), or only antheridium formation (*Campyloneurum phyllitidis, Microgramma heterophylla, Phymatosorus scolopendria*), or neither response (*Campyloneurum angustifolium, Lepisorus thunbergianus*). These results suggest two possibilities. First, antheridiogen may be different from germinin, and the latter may be short-lived or lose activity during the extraction process. Second, antheridiogen and germinin may be the same thing, but the threshold concentration for promoting antheridium formation is lower than that for substituting for the light requirement for spore germination. On the other hand, secretions of *P. aquilinum* gametophytes promoted both antheridium formation and spore germination in darkness in Polypodiaceae (except in *P. aureum* in which only antheridium formation was promoted), whereas culture extracts of *P. aquilinum* either promoted both functions in Polypodiaceae (*C. phyllitidis, M. heterophylla, P. pellucidum, P. scolopendria*), or only induced spore germination in darkness (*C. angustifolium, L. thunbergianus*). These results suggest that antheridiogen and germinin are both stable in extraction and are possibly the same chemical, at least in *P. aquilinum*, but the threshold concentration for antheridium
formation may be higher or lower than that for inducing spore germination in darkness depending on the species.

**Antheridiogen vs. inhibitor**

In our antheridiogen experiments, gametophytes on treated media were somewhat smaller than those on control media (Fig. 6). Usually only spathulate or small cordate gametophytes developed on media containing extract of gametophyte cultures and only few celled filaments to spathulate stages of prothalli developed on media already supporting other mature gametophytes. Only small sizes were attained regardless of whether they bore antheridia or remained asexual. Naf (1956) suggested that in gametophytes subjected to antheridiogen, potential vegetative growth is diverted to antheridium production. However, this cannot explain the small asexual gametophytes. We suspected the activity of another growth inhibiting substance. To test this hypothesis, we sowed spores of the Polypodiaceae on inverted and cleared agar blocks on which mature gametophytes of *Anemia phyllitidis* were growing. *Anemia phyllitidis* produces antheridiogen $A_{An}$ which is inactive in families outside the Schizaeaceae. All gametophytes of Polypodiaceae growing on these agar blocks developed only filaments or spathulate stages and remained asexual during three months of culture. These results indicates that growth inhibitors may be universal and chemically/physiologically more closely related than antheridiogens among fern gametophytes. It seems that Polypodiaceae gametophytes produce their own antheridiogen as well as inhibitor, and respond to both of them. On the other hand, the inhibitor produced from *A. phyllitidis* inhibited growth of Polypodiaceae gametophytes but antheridiogen $A_{An}$ did not promote antheridium formation by Polypodiaceae gametophytes. An alternative hypothesis is that antheridiogen functions in both antheridium promotion and gametophyte growth inhibition, but the efficiency of these two functions is different depending on the species. In either case, the statement that "antheridiogens actually affect size, and size influences sex
expression” (Korpelainen 1994) does not adequately describe the situation in the species tested here.

Genetic load

Genetic load was estimated from isolated-spore and isolated-gametophyte cultures. Genetic load varied from 3% to 100% (Table 5). Genetic load estimated from isolated-spore cultures was not significantly different from that obtained from isolated-gametophyte cultures, except for *Campyloneurum phyllitidis* in which the genetic load of isolated-spore cultures was much greater than that of isolated-gametophyte cultures (Table 5). This apparent elevation of genetic load in isolated-spore cultures in *C. phyllitidis* may be due to failure of the gametophytes to obtain functional bisexuality rather than expression of recessive lethals (chapter 2). Pairing of gametophytes from different sporophytes in *C. phyllitidis* failed to relieve the sporophyte suppression observed in isolated gametophyte cultures. This suggests that the genetic load is being expressed in gamete development (Klekowski 1971). If non-functional eggs or archegonia are being produced, pairing of gametophytes would be of no consequence (chapter 2).

Mating/breeding system and ploidy

The presence of high genetic load indicates that sporophytes of six of the ten taxa are mainly produced through intergametophytic mating. Four taxa expressed very low genetic loads indicating that sporophytes are produced mainly by intragametophytic selfing (Table 6). Both intra- and intergametophytic matings are possibly indicated for *Campyloneurum*. The mating system of *C. angustifolium* may depend on ploidy level, with tetraploids reproducing by intragametophytic selfing, and diploids by intergametophytic mating (chapter 1). Intergametophytic mating by *C. phyllitidis* may be influenced by an antheridiogen system (chapter 2). Antheridiogen interaction occurring temporarily at early gametophyte stages may promote successful intragametophytic selfing by increasing bisexual development. On the
Table 5. Genetic load of some epiphytic ferns, estimated from mono-spore cultures (spore) and mono-gametophyte cultures (gametophyte).

<table>
<thead>
<tr>
<th>Species</th>
<th>Load²</th>
<th>Spore</th>
<th>Gametophyte</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campyloneurum angustifolium (1)</td>
<td>16 (22)</td>
<td>12 (10)</td>
<td>14 (11)</td>
<td></td>
</tr>
<tr>
<td>C. angustifolium (2)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>C. phyllitidis (1)</td>
<td>74 (15)</td>
<td>38 (10)</td>
<td>56 (16)</td>
<td></td>
</tr>
<tr>
<td>C. phyllitidis (2)</td>
<td>83 (18)</td>
<td>31 (12)</td>
<td>57 (20)</td>
<td></td>
</tr>
<tr>
<td>Lepisorus thunbergianus</td>
<td>94 (12)</td>
<td>96 (8)</td>
<td>95 (10)</td>
<td></td>
</tr>
<tr>
<td>Microgramma heterophylla</td>
<td>100</td>
<td>98 (6)</td>
<td>99 (4)</td>
<td></td>
</tr>
<tr>
<td>Phlebodium aureum (1)</td>
<td>19 (14)</td>
<td>13 (8)</td>
<td>16 (11)</td>
<td></td>
</tr>
<tr>
<td>P. aureum (2)</td>
<td>7 (14)</td>
<td>3 (6)</td>
<td>5 (10)</td>
<td></td>
</tr>
<tr>
<td>Phymatosorus scolopendria</td>
<td>12 (11)</td>
<td>10 (9)</td>
<td>11 (10)</td>
<td></td>
</tr>
<tr>
<td>Polypodium pellucidum</td>
<td>100</td>
<td>96 (5)</td>
<td>98 (3)</td>
<td></td>
</tr>
<tr>
<td>E. callifolium</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>E. crassifolium</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

¹ C. angustifolium (1) & (2), C. phyllitidis (1) & (2), and Phlebodium aureum (1) & (2) are from two different sporophytes respectively.
² Numbers in parentheses are standard deviation.
<table>
<thead>
<tr>
<th>Species</th>
<th>Mating system</th>
<th>Breeding system</th>
<th>Ploidy^5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campyloneurum angustifolium (1)^1</td>
<td>Intra</td>
<td>-</td>
<td>P (Evans 1963)</td>
</tr>
<tr>
<td>C. angustifolium(2)^1</td>
<td>Inter</td>
<td>-</td>
<td>D (Evans 1963)</td>
</tr>
<tr>
<td>C. phyllitidis</td>
<td>Intra^3</td>
<td>-</td>
<td>P (Isozyme) (Evans 1963)</td>
</tr>
<tr>
<td>Lepisorus thunbergianus</td>
<td>Inter</td>
<td>-</td>
<td>P (Isozyme)</td>
</tr>
<tr>
<td>Microgramma heterophylla</td>
<td>Inter</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phlebodium aureum</td>
<td>Intra</td>
<td>-</td>
<td>P (Isozyme)</td>
</tr>
<tr>
<td>Phymatosorus scolopendria</td>
<td>Intra</td>
<td>-</td>
<td>P (Tsai &amp; Shieh 1983)</td>
</tr>
<tr>
<td>Polypodium pellucidum</td>
<td>Inter</td>
<td>-</td>
<td>D (Manton 1951)</td>
</tr>
<tr>
<td>Elaphoglossum alatum</td>
<td>-</td>
<td>O</td>
<td>D (Isozyme) (Wagner unpub.)</td>
</tr>
<tr>
<td>E. callifolium</td>
<td>Inter</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. crassifolium</td>
<td>Inter</td>
<td>O</td>
<td>D (Isozyme) (Wagner unpub.)</td>
</tr>
<tr>
<td>E. paleaceum</td>
<td>-</td>
<td>-</td>
<td>P (Isozyme) (Wagner unpub.)</td>
</tr>
</tbody>
</table>

1 Campyloneurum angustifolium (1) & (2) are from two different sporophytes.
2 Estimated from average load of isolated-spore cultures and isolated-gametophyte cultures (since no significant difference of genetic load between them) unless specifically indicated. Inter = Intergametophytic mating, Intra = Intragametophytic selfing.
3 Estimated from isolated-gametophyte cultures.
4 Estimated from isozyme patterns. O = Outbreeding.
5 Estimated from isozyme patterns (Isozyme) and chromosome number counting (Reference). D = Diploid, P = Polyploid.
other hand, a relatively persistent antheridiogen effect may increase the number of male
gametophytes and thus the opportunity for intergametophytic mating. Antheridiogen
apparently serves the latter function in *Lepisorus thunbergianus*, *Microgramma heterophylla*,
and *Polypodium pellucidum*.

Isozyme electrophoresis has been used to estimate the breeding system and ploidy
level in ferns (Haufler 1987). Most species we studied are polyploid (Table 6) and the
estimation of their breeding system by electrophoresis is not possible. The isozyme data for
*E. crassifolium* indicating outbreeding are in accordance with the high genetic load observed
for this species in isolated gametophyte cultures. However, absence of antheridiogen in this
species indicates that genetic load alone can maintain an intergametophytic mating system.

In general, diploid species produce sporophytes through intergametophytic mating
(Masuyama and Watano 1990). *Polypodium pellucidum* and one source of *Campyloneurum
angustifolium*, *Elaphoglossum alatum*, and *E. crassifolium* fit this pattern. Although
polyploid species often produce sporophytes through intragametophytic selfing, this is not
the case in *Lepisorus thunbergianus*, which maintains a very high genetic load. This is
evidenced in the very high number of recessive lethals expressed even in paired
gametophytes (intergametophytic selfing (chapter 3)).

Despite their high chromosome numbers, most ferns with chromosome numbers basal
for their genus behave genetically like diploids. Possibly functional diploidy has been
achieved through silencing of ancient gene duplicates (Haufler 1987; Soltis and Soltis 1989).
In the four polyploid (relative to the base number for the genus) species examined by
electrophoresis, isozyme patterns revealed that a high proportion of locus pairs were
characterized by only one allele (Table 7). This is perhaps because of high levels of
homozygosity in the sampled populations. Alternatively, this may be due to gene silencing in
process.
Table 7. Percentage of locus pairs with only a single allele expressed in polyploid species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Total detected locus pair #</th>
<th>% of a single allele expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campyloneurum phyllitidis</em></td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td><em>Phleobodium aureum</em></td>
<td>22</td>
<td>50</td>
</tr>
<tr>
<td><em>Lepisorus thunbergianus</em></td>
<td>19</td>
<td>42</td>
</tr>
<tr>
<td><em>Elaphoglossum paleaceum</em></td>
<td>17</td>
<td>53</td>
</tr>
</tbody>
</table>

**Conclusion**

The long-lived and clone-forming habit of gametophytes of epiphytic ferns has been hypothesized to be of adaptive value in competing with bryophytes and increasing the opportunity for gametophytes to find favorable space and time for sporophyte production (Dassler 1995). Dassler stated this habit “... allows a fern gametophyte to physically migrate to a microsite favorable for growth and reproduction, as well as to survive until favorable microsite conditions occur, and to persist until the arrival of second migrant”. These clonal, long-lived gametophytes continuously form gametangia on their new proliferations, and thus increase number of gametes as well as lengthen the opportunity for fertilization. If we consider antheridiogen function, the promotion of gametophyte interaction by clone-forming and long-lived habits is even more obvious, including interaction with previously buried spores. However, our observations on species of Polypodiaceae and *Elaphoglossum* have been performed in the laboratory. The degree to which these observations relate to gametophyte form and interaction in nature is unknown.

Antheridiogen has been considered to increase the number of male gametophytes, thus increasing the opportunity of outcrossing and maintenance of a high genetic load. The correlation between antheridiogen function and high genetic load observed for many species (Schneller *et al.* 1990) does not hold for all the ferns studied here. Some polyploid species produce antheridiogen but are frequent inbreeders with low genetic load. In these,
antheridiogen may increase the opportunity for occasional outcrossing by inducing unisexual gametophytes and thus partially compensate for the low genetic diversity caused by inbreeding (Masuyama and Watano 1990). Alternatively, antheridiogen production and response in these polyploid species may be a vestige inherited from their diploid ancestors. On the other hand, in the presumed polyploid *Lepisorus thunbergianus*, antheridiogen, outcrossing, and high genetic load have been maintained despite polyploidy.

How widely antheridiogen is distributed is still unclear (Schneller *et al.* 1990). The demonstration of antheridiogen production and response in the Polypodiaceae species tested indicates that antheridiogen is more widely distributed than previously thought. Other species in the Polypodiaceae will likely be found to possess antheridiogen systems when reexamined using the more sensitive method of spores sown on agar adjacent to growing mature gametophytes.

Another function of antheridiogen (or germinin) is to induce spore germination in darkness. For epiphytic species, fern spores are possibly deposited very deep in bryophyte mats where light intensity may be insufficient for spore germination. Through the antheridiogen influence, these "buried" spores may be induced to germinate, and the resultant gametophytes could precociously produce antheridia and release active sperms which could interact with older gametophytes. This model has been proposed previously (Voeller 1971; Schneller *et al.* 1990; Haufler and Welling 1994) for terrestrial ferns, and may fit epiphytic species as well. Operation of this system in nature needs to be demonstrated. Since gametophytes of different species may be randomly mixed together in nature, the antheridiogen effect between different species also needs investigation. Whether antheridiogen functions primarily to promote outcrossing, thus increasing genetic diversity or whether it also functions to reduce sporophyte competition by promoting formation of unisexual gametophytes (Willson 1981) is also an issue worthy of study.
Whether species are outbreeders or inbreeders, their breeding behavior is adaptive to establishment and survival of individuals of that species. In general, inbreeding may be advantageous to developing new populations following long range spore dispersal where gametophytes are likely to be derived from single isolated spores. Outbreeding has the advantage of retaining genetic diversity. High genetic load carried by outbreeders tends to maintain that mating system. Breeding systems and genetic load interwoven with factors of morphology, growth habit, antheridiogen and environmental parameters maintain successful reproduction. Comprehensive research of this type is limited, especially in epiphytic ferns (Werth and Cousens 1990), but feasible and valuable, as demonstrated by this study of gametophyte morphology and reproductive behavior of epiphytic species. Continued studies of fern gametophyte morphology, growth habit, reproductive behavior, and the adaptation among them in both laboratory and nature are needed to fully understand fern biology.

**Literature cited**


