Phylogeography of eastern leatherwood (*Dirca palustris* L.) resolved by chloroplast sequencing and microsatellite genotyping

by

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ABSTRACT

The broad distribution of eastern leatherwood (*Dirca palustris* L.), from Florida to Canada, contrasted with its patchy restriction to rich, mesic forests and the paucity of vectors for dispersal of its seeds, represents a classic example of Reid's paradox of plant migration. It long has been presumed that temperate forest species of eastern North America persisted in southern glacial refugia near the Gulf Coast, after which many dispersed nearly 2000 km to contemporary northern range limits. The rates of migration necessary to account for present distributions exceed those suggested by contemporary observations of seed dispersal. I used ecological niche modeling, chloroplast sequencing, microsatellite genotyping, and phenotypic investigations to answer questions about the historical and contemporary distribution of populations and genes of *D. palustris* across the landscape of eastern North America. Ecological niche modeling supported my hypothesis that *D. palustris* persisted within mid-latitudinal refugia during the Last Glacial Maximum, and the distribution of chloroplast haplotypes supported the prehistoric presence of individuals at such latitudes. Deep diversification of chloroplast haplotypes indicated a complex history of population isolation, migration, and admixture over Pleistocene glaciations. I used next-generation sequencing for *de novo* isolation of highly polymorphic microsatellite loci from *D. palustris*. Microsatellite genetic analyses provided further evidence for mid-latitudinal refugia. Results from both markers were indicative of general dispersal limitation with instances of long-distance dispersal, especially to the Upper Midwest and Great Lakes Region; I suggest that an active vector likely was important for post-glacial range expansion of *D. palustris*. I provide genetic and phenotypic evidence for a new subspecies of *D. palustris* that is restricted in distribution to

the Southeastern U.S. The putative subspecies has white pubescence on its bud scales, unlike the brown described for the species, and calyx tubes that are shorter and with wider limbs than those of *D. palustris* elsewhere.

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Dissertation Organization

Three manuscripts and a chapter describing evidence for a new subspecies of *D. palustris* are presented in this dissertation. Chapter 2 is a manuscript of original research in which I used ecological niche modeling and chloroplast sequencing to answer questions about the distribution and migration of *D. palustris* in response to Pleistocene climate oscillations, particularly changes since the Last Glacial Maximum. This manuscript is intended for submission to the *Journal of Biogeography* and has been formatted for that journal. Chapter 3 is a manuscript detailing the isolation and characterization of microsatellite genetic markers from *D. palustris* by next-generation sequencing generated by using the Illumina HiSeq 2500 platform. To my knowledge, this platform has not been used before to isolate microsatellite markers from plants. The manuscript is intended for publication in *AJB Primer Notes & Protocols in the Plant Sciences* and has been formatted for submission to that journal. Chapter 4 presents a manuscript of original research in which I used microsatellite genetic markers to investigate the population genetics of *D. palustris*, and compared results to those obtained from chloroplast sequencing. This manuscript is intended for publication in the *American Journal of Botany* and has been formatted for that journal. Chapter 5 presents evidence for a new subspecies, *Dirca palustris* L. ssp. *nivea* Peterson & Graves, from the southeastern United States. The contents of Chapter 5 are intended for publication following addition of a formal description for the putative subspecies, designation of type specimens, and description of examined specimens. I present general conclusions and an interpretive summary for my work. Herbarium voucher labels for populations used in these studies are shown in an appendix.

Introduction

One of the most compelling sources of information about predicted responses of taxa to climate change is the growing body of data on the responses of plants to past changes in climate. Plants indigenous to eastern North America exist where they do because their ancestors survived a world of rapid climate changes with the advance and retreat of continental glaciers, the most recent of which reached its southernmost extent ~20,000 years ago. In recent decades, questions about patterns and processes of vicariance and persistence of temperate taxa during glacial maxima, and dispersal and range expansion during interglacials, have received increased attention.

Phylogeography, the study of factors that govern the distribution of lineages over space and time, addresses questions that vary in scale from the evolutionary origins of entire taxa, to the distribution of lineages across the landscape, to contemporary genetic structure among populations (Avise 2000). A comprehensive phylogeographical approach is an excellent means for studying questions about persistence, dispersal, and gene flow among components of eastern North America's flora. Although it is a relatively young discipline (ca. 1987), phylogeography has contributed much to our knowledge of the histories and distributions of gene lineages during periods of Pleistocene climate change (Avise 2000). However, more work needs to be conducted on late-Pleistocene distributions and post-glacial dispersals of terrestrial plants of eastern North America, as the majority of research (~80%) has focused on animals (Soltis et al. 2006; Beheregaray 2008). Unlike animals, which often are mobile, plants provide the opportunity to investigate the climate-mediated responses of sessile taxa, for which individuals are unable to relocate to more favorable environments as climates change. A particularly relevant controversy in the study of plant dispersal and post-glacial

phylogeography is Reid's paradox of plant migration (Clark et al. 1998), the observation that the migration rates of taxa inferred from the northern extent of present-day distributions outpace rates considered realistic based on contemporary observations of seed dispersal.

During the Last Glacial Maximum, the Laurentide ice sheet covered vast expanses of land at northern latitudes of North America. This ice covered much of the Upper Midwest and New England, and cooling climates presumably shifted tundra into regions previously occupied by boreal forests, shifted boreal forests southward to mid-latitudes, and shifted temperate forests south to coastal regions south of ~34°N (Delcourt and Delcourt 1998; Soltis et al. 2006). Boundaries delineating vegetation assemblages during this time are not without considerable dispute, and parts of the southeastern U.S. may have been composed of a heterogeneous assortment of boreal taxa and temperate species (Jackson et al. 2000). Following the Last Glacial Maximum, receding ice left northern latitudes open for colonization from the south, and temperate forest species occupying northern latitudes today represent descendants of post-glacial migrants from refugia to the south.

The northern distribution of many temperate species, coupled with contemporary observations suggesting low to modest capacities for dispersal of their seeds, is the essence of Reid's paradox of plant migration (Clark et al. 1998). It long has been presumed that temperate forest elements of eastern North America persisted in low-latitudinal glacial refugia near the Gulf Coast, after which many expanded in range by nearly 2000 km to contemporary northern range limits at high latitudes. The rates of migration necessary to account for present distributions of many temperate forest taxa are considered greater than contemporary observations of seed dispersal would suggest (Clark et al. 1998). At least two potential solutions have been suggested to resolve this paradox. The first solution is that historical long-

distance seed dispersal may have occurred more frequently than present-day observations would suggest. The second is that many temperate species were present in one or more cryptic refugia within several hundred kilometers of the Laurentide ice sheet during the Last Glacial Maximum, considerably reducing the rates of migration necessary to account for present northern range limits.

The presence of cryptic mid-latitudinal refugia of temperate deciduous taxa in regions like the Ozark Ecoregion and the Appalachian Basin of West Virginia, Kentucky, and Tennessee, has received support as a plausible solution to this paradox. Although once viewed as an unlikely hypothesis, genetic evidence has accumulated to support such refugia harboring low-density populations of temperate tree species, some of which seem to have been as close as 500 km from the Laurentide ice sheet (McLachlan et al. 2005; Soltis et al. 2006; Gonzales et al. 2008). The primary implication of northern refugia is that post-glacial migration rates of many temperate species may not be as great as those inferred from present range limits because these inferences are based on an assumption of southern (e.g., Gulf Coastal) refugia. Such a conclusion may have strong consequences for our models and expectations of plant responses to changing climates.

Despite progress on temperate forest phylogeography in eastern North America, more studies should investigate a diversity of taxa currently understudied. Most studies on the phylogeography of temperate woody plants have focused on community-dominating species that are important to forestry, wind-pollinated, and exhibit seed characteristics facilitating long-distance dispersal (Jaramillo-Correa et al. 2009). This leaves a critical gap in our knowledge of the responses of subdominant taxa, such as understory plants that are uncommon, insect-pollinated, or lack an obvious capacity for seed dispersal. From a phylogeographical

perspective, the distinction between dominant, wind-pollinated taxa with obvious means of dispersal, and less common, insect-pollinated taxa, or taxa with apparently limited avenues for seed dispersal, is crucial. First, much more is known about the paleodistributions and postglacial range expansions of dominant wind-pollinated taxa, as they tend to produce abundant pollen, which leaves a substantial imprint on the pollen record of the late Pleistocene and Holocene (Jackson et al. 2000); insect-pollinated plants produce comparatively little pollen and do not generally appear in palynological studies (Faegri and Iversen 1989). Second, plants that routinely experience long-distance dispersal are most likely to effectively track climate change; taxa lacking mechanisms for dispersal are at risk of decline if distributions lag behind suitable climates (Zhu et al. 2012).

Given the need to broaden investigations to include understudied taxa, I have identified a taxon for which a thorough study is likely to contribute in a novel way to the existing literature of eastern North America's phylogeography. Eastern leatherwood (*Dirca palustris* L.) exemplifies a broadly distributed, yet uncommon, taxon absent from the historical pollen record, and for which no means for long-distance seed dispersal has been observed (Ward and Horn 1998; Peterson and Graves 2011). Because of the scarcity of information about how such components of eastern North America's forests persisted, colonized, and exchanged genes during periods of rapid climate change, this work can make an important addition to the growing body of literature on the phylogeography of eastern North America's understudied flora.

Distribution and Ecology of Eastern Leatherwood

Dirca is the sole North American representative of the Thymelaeaceae, a cosmopolitan

family of plants. The genus comprises four species of woody shrubs, all indigenous to forests of North America. *Dirca palustris* (eastern leatherwood) is present in eastern North America; *D. occidentalis* Gray is endemic to six counties near the San Francisco Bay; and *D. mexicana* Nesom & Mayfield is known from one population in the Sierra Madre Oriental mountain range in Tamaulipas, Mexico (Nesom and Mayfield 1995). Floden et al. (2009) recently described a fourth species, *Dirca decipiens* Floden & Mayfield, from several populations at the western range limits of *D. palustris* in Kansas and Arkansas.

The distribution of *D. palustris,* the most wide-ranging member of the genus, extends from New Brunswick west to North Dakota, and south to Oklahoma and Florida (Steyermark 1963; Gleason 1968). Across this distribution, *D. palustris* generally occurs infrequently but can be locally abundant (Nevling 1962; Godfrey 1988; Ward and Horn 1998). It is found in rich, mesic forests on stream banks and forested bluffs, and often on north- or east-facing slopes (Steyermark 1963; Gleason 1968). Despite its broad distribution, active vectors for its seed dispersal have not been identified (Ward and Horn 1998; Peterson and Graves 2011), and most fruits seem to fall to the forest floor beneath maternal plants, where they become incorporated into the leaf litter. The local distribution of *D. palustris* tends to be clumped, with seedlings abundant near maternal plants and often absent in other apparently suitable sites (Peterson and Graves 2011). The species is uncommon in the southern half of its distribution, where it is restricted to cool, moist niches (Nevling 1962; Ward and Horn 1998). Populations in the South are Pleistocene relicts (Floden et al. 2009), which may be in danger of extinction due to climate changes since the Last Glacial Maximum. In contrast, the species is somewhat more abundant at higher latitudes, and sometimes is a dominant member of the shrub community in northern hardwood forests in the Upper Great Lakes Region (Schulz et al. 2004).

The broad range of *D. palustris*, in contrast with its patchy restriction to rich, mesic forests and the paucity of vectors for dispersal of its seeds, represents a classic example of Reid's paradox of plant migration. Northward migration of eastern leatherwood following the Last Glacial Maximum has resulted in a present distribution that extends nearly 2000 km north of traditionally cited locations of coastal refugia for temperate deciduous species (McLachlan et al. 2005; Soltis et al. 2006). Given the present distribution of the species, the average rate of migration for eastern leatherwood from traditional Gulf Coastal refugia would have to exceed rates estimated for dominant forest trees known to disperse seeds by wind and animal vectors (McLachlan et al. 2005).

Peterson et al. (2009) identified a population of *D. palustris* in Florida that differed phenotypically from other populations and from descriptions of the species. If seed dispersal of *D. palustris* historically was limited, these phenotypically unique populations in the Southeast are not likely sources of propagules that colonized northern latitudes. Instead, these populations may represent an isolated lineage predating the Last Glacial Maximum and warranting recognition as a distinct taxonomic, and conservation, unit. Alternatively, migration of eastern leatherwood to northern latitudes could have occurred via transport of propagules from southern refugia by vectors not recognized. Identifying the refugial patterns of persistence and postglacial dispersal of eastern leatherwood represents a significant contribution to the literature, with implications for conservation of unique lineages.

Objectives

My objectives were to test several hypotheses about the phylogeography of eastern North American forests by studying *D. palustris*, a shrub that is both characteristic of northern

hardwood forests and persistent in relictual populations in the Southeast. I sought to clarify the means by which this shrub with no presence in the pollen record and without obvious agents for long-distance dispersal persisted, migrated, and exchanged genes across the landscape of eastern North America during climate oscillations and since the Last Glacial Maximum. I used chloroplast sequencing, ecological niche modeling, microsatellite genotyping, and phenotypic investigations to address the following objectives:

1. Identify putative locations of refugia for *D. palustris* during the Last Glacial Maximum;
2. Determine whether populations in northern latitudes were founded by propagules from Gulf Coastal refugia or from mid-latitudinal refugia as close as several hundred kilometers from the Laurentide ice sheet;
3. Identify the extent to which landscape features like the Appalachian Mountains and the Mississippi River impeded gene flow across the landscape; and
4. Identify whether phenotypically distinct populations in the Southeast warrant taxonomic or conservation attention.

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CHAPTER 2. CHLOROPLAST PHYLOGEOGRAPHY OF *DIRCA PALUSTRIS* L. INDICATES DEEP PLEISTOCENE DIVERSIFICATION AND CONFIRMS MID-LATITUDINAL REFUGIA DURING THE LAST GLACIAL MAXIMUM IN EASTERN NORTH AMERICA

A manuscript intended for submission to the *Journal of Biogeography*

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ABSTRACT

Aim To assess evidence for the existence of mid-latitudinal temperate refugia for *Dirca palustris* L. during the Last Glacial Maximum (LGM) and date divergence events within the genus *Dirca.*

Location Eastern North America and California

Methods We conducted ecological niche modelling (ENM) and sequenced five noncoding regions of the chloroplast genome from 310 plants among 104 populations, including individuals from the four described species of *Dirca.* Molecular diversity within the genus was assessed, and we used a Bayesian approach to identify relationships among individuals and estimate timing of lineage splits within *D. palustris* and among the species. Spatial genetic structure within *D. palustris* was identified with several techniques, including analysis of molecular variance (AMOVA) and Monmonier's algorithm.

Results ENM indicated suitable LGM climate in the Gulf Coastal Region and as far north as

northern Arkansas (Ozark Plateaus Province), West Virginia and eastern portions of Tennessee (Appalachian Plateaus Province), and the Piedmont and Coastal Plain Provinces of the Carolinas and Virginia. Sequence variation was extensive and indicated deep lineage splits within *D. palustris*. Numerous haplotypes, many population-specific, were identified in mid-latitudinal regions. AMOVA indicated that the Appalachian Mountain Range was a barrier to seed dispersal, and Monmonier's algorithm supported the presence of mid-latitudinal (35-40 °N) genetic discontinuities.

Main conclusions ENM and genetic evidence support the existence of mid-latitudinal refugia within several hundred kilometers of the Laurentide ice sheet during the LGM. Deep Pleistocene diversification exists within *D. palustris* along with weak geographic fidelity of haplotypes, likely the product of repeated isolation, differentiation, and secondary contact during Quaternary glacial cycles. *Dirca occidentalis* (now restricted to California) became isolated during the late Miocene, and eastern *Dirca* diversified as glaciations increased in amplitude and frequency during the middle Pleistocene. Our findings demonstrate that temperate understorey shrubs likely accompanied temperate trees of eastern North America in mid-latitudinal refugia during the LGM.

Keywords

Climate change, cryptic refugia, disjunction, eastern leatherwood, ecological niche modeling, migration, Reid's paradox, seed dispersal.

Introduction

Plants indigenous to eastern North America exist where they do because their ancestors survived the climate fluctuations of the Quaternary ice age and the repeated advance and retreat of continental glaciers, the most recent of which reached its southernmost extent ~20,000 years ago (Comes and Kadereit 1998; Hewitt 2004). Ice periodically covered much of Canada and the northeastern and upper midwestern parts of the U.S., shifting boreal forests southward to mid-latitudes, and temperate forests further south towards coastal regions below ~34°N (Delcourt and Delcourt 1998; Soltis et al. 2006). Boundaries delineating vegetation assemblages during the LGM are disputed, and portions of the eastern U.S. may have been composed of a heterogeneous assortment of boreal and temperate taxa (Jackson et al. 2000). Ice receding after the last glacial maximum (LGM) left land at northern latitudes open for colonization, and temperate forest species occupying northern latitudes today represent descendants of post-glacial migrants from the south, often presumed to be from deeply southern refugia (McLachlan et al. 2005).

Phylogeographers have begun to answer questions about the evolutionary histories of plant taxa and to identify both putative refugial locations of various taxa during the LGM and patterns of range expansion during the current interglacial period (Avise 2000; Soltis et al. 2006). More knowledge of late-Pleistocene distributions and post-glacial dispersals of terrestrial plants is needed, as ~80% of phylogeographic studies have focused on mobile animals (Soltis et al. 2006; Beheregaray 2008). In contrast to animals, terrestrial plants do not relocate during their lifetimes in response to unfavorable environments, and migration rates of taxa inferred from the northern extent of present-day distributions often exceed rates considered realistic based on contemporary observations of seed dispersal (Clark et al. 1998).

Resolution of competing solutions to this observation, known as Reid's paradox, will improve our understanding of how sessile taxa are responding to past and contemporary climate change.

Cryptic mid-latitudinal refugia (35-40 °N) that are farther north than inferred by palynological studies may be a solution to this paradox. There is genetic evidence for such refugia harboring low-density populations of temperate tree species as close as 500 km from the Laurentide ice sheet (McLachlan et al. 2005; Soltis et al. 2006; Gonzales et al. 2008). The primary implication of mid-latitudinal refugia is that post-glacial migration rates of many temperate species may be lower than those inferred from a traditional assumption of refugia located exclusively in the Gulf Coastal Region (McLachlan et al. 2005; Pearson 2006). An alternative solution to Reid's paradox is that distances of seed dispersal routinely have exceeded those inferred by contemporary observations because infrequent, long-distance dispersal is difficult to document (Clark 1998; Clark et al. 1998).

Plant taxa absent from the literature of molecular phylogeography and palaeo-distributions merit attention. Most studies on the phylogeography of temperate woody plants have focused on community-dominating species prevalent throughout their ranges, particularly species that are important to forestry, are wind-pollinated, and/or have readily dispersed seeds (McLachlan et al. 2005; Jaramillo-Correa et al., 2009). However, the responses of understorey shrubs that are distributed sporadically, are insect-pollinated, or lack an obvious capacity for seed dispersal have not been widely studied. Distinctions among different life histories are crucial from a phylogeographic perspective. First, much more is known about the palaeodistributions and postglacial range expansions of dominant wind-pollinated taxa that tend to produce abundant, mobile pollen that imprinted the pollen record of the late Pleistocene and Holocene (Jackson et al. 2000); insect-pollinated plants produce comparatively little pollen

and are therefore scarce in palynological records (Faegri and Iversen 1989). Second, migration of taxa with capacities for long-distance dispersal is likely to be near equilibrium with the migration of suitable climate. In contrast, the migration of taxa lacking obvious dispersal mechanisms may lag behind the migration of suitable climate due to dispersal limitation (Zhu et al. 2012).

Dirca palustris L. (eastern leatherwood) is a shade-tolerant shrub characteristic of climax forest communities (Schulz et al. 2004). It persists in dark understories of rich, late-successional *Acer saccharum* Marsh. - *Fagus grandifolia* Ehrh – *Quercus rubra* L. forests throughout much of the eastern half of its distribution. In its western distribution, *D. palustris* usually occupies *Acer saccharum* - *Quercus rubra* forests, although it occurs under *Acer nigrum* in some portions of its distribution and under *Quercus macrocarpa* Michx. at the northwestern limit of its distribution in North Dakota (Schulz et al. 2004; Peterson and Graves 2011). Despite its broad northern distribution extending into southern Canada, there is no evidence for mechanisms of long-distance seed dispersal (Ward and Horn 1998; Peterson and Graves 2011). The species is absent from the historical palynological record, likely because its insect-pollinated flowers produce few pollen grains with limited mobility (Faegri and Iversen 1989). Because of the dearth of information about how plant taxa with these characteristics persisted, migrated, and colonized new regions during periods of rapid climate change, *D. palustris* is an intriguing addition to the growing body of literature on the phylogeography of eastern North American flora.

Four species of *Dirca* have been described, all indigenous to North America. *Dirca occidentalis* Gray is endemic to six counties around the San Francisco Bay in California (Graves and Schrader 2008). *Dirca mexicana* Nesom & Mayfield, regarded as *D. palustris*

when first collected in 1970 (F.G. Medrano, ASU specimen image ASU0050618), is known from one population in the Sierra Madre Oriental of Tamaulipas, Mexico (Nesom and Mayfield 1995). *Dirca decipiens* Floden & Mayfield was described recently from several populations in Kansas and Arkansas that had been identified originally as *D. palustris* (Floden et al. 2009). *Dirca palustris* is the most broadly distributed species, occurring from Nova Scotia, west to North Dakota, and south to Oklahoma, Louisiana, and Florida (Peterson et al. 2009). In the northern half of its distribution, where it is most common, *D. palustris* nevertheless usually occurs in small, localized populations of tens to hundreds of individuals, with populations often separated by many kilometers. An exception to this pattern seems to be in the Upper Great Lakes Region of northern Wisconsin and the Upper Peninsula of Michigan, where the species can be prevalent (Schulz et al. 2004). Near the southern limit of its range, *D. palustris* is infrequent in cool, moist scattered niches (Nevling 1962; Steyermark 1962; Ward and Horn 1998; Williams 2009). Southeastern populations likely represent Pleistocene relicts in decline due to climate changes since the LGM (Floden et al. 2009). We have speculated (Peterson et al. 2009) that several populations in northern Florida, where the species is endangered (Wunderlin and Hansen 2008), and at least one population in Alabama, may be genetically distinct remnants of a Gulf Coastal Pleistocene refugium. White pubescence on bud scales is characteristic of plants in these populations and is distinct from the brown pubescence typical of this species (Peterson et al. 2009; 2011).

Northward migration of eastern leatherwood following the LGM has produced a present distribution that extends nearly 2000 km north of traditionally cited locations of refugia for temperate deciduous species in the southern Coastal Plain Province (McLachlan et al. 2005; Soltis et al. 2006). Given its contemporary distribution, the average rate of migration for *D.*

palustris from traditional Gulf Coastal refugia would need to match or exceed rates estimated for several dominant woody species for which seeds readily disperse by wind and animal vectors (Iverson et al. 2004; McLachlan et al. 2005; Ordonez and Williams 2013). Fruits of *Dirca* lack traits favouring dispersal; most fall to the forest floor directly under maternal plants and do not produce colour signals of ripeness to attract frugivores (Graves 2008; Floden et al. 2009). If seed dispersal has been limited historically, then populations in the southeastern U.S. are not likely the source of propagules that have colonized northern latitudes. Alternatively, migration of eastern leatherwood to northern latitudes could have occurred via transport of propagules from exclusively southern refugia by unknown vectors (Pearson 2006). Identifying the refugial patterns of persistence and postglacial dispersal of *D. palustris* should help resolve the paradoxical nature of its present distribution.

Our objective was to contribute information about the phylogeography of eastern North American forests by studying where *D. palustris*, a shrub with no presence in the pollen record and without obvious agents for long-distance dispersal, persisted during the LGM and how it expanded its distribution during the present interglacial period. Specifically, we used ecological niche modelling and phylogeographic analyses based on chloroplast sequences to answer the following questions about *D. palustris*: 1) Where did putatively suitable climates exist during the LGM? 2) How much haplotypic diversity exists within *D. palustris*? 3) Is there deep genetic divergence of maternal lineages indicative of a complex Pleistocene history? 4) Does haplotypic evidence support the persistence of populations at middle latitudes during the LGM, such as in the Ozark Plateaus Province, the Interior Lowland Plateaus Province, or the Appalachian Plateaus Province? 5) From which putative refugia did propagules responsible for colonization of northern latitudes originate? 6) Do phenotypically distinct populations in the southeastern

U.S. belong to a distinct phylogenetic lineage? 7) How distinct are *Dirca* that have been ascribed to different species? 8) When did major intrageneric diversification events occur?

Materials and Methods

Niche modelling and LGM distribution

We constructed an ecological niche model (ENM) and projected it onto the climatic conditions of the LGM to test the hypothesis that *D. palustris* persisted in mid-latitudinal refugia. We initially included 114 populations of *D. palustris* where GPS coordinates were recorded during site visits. The model was constructed with MaxEnt 3.3.3 (Phillips et al. 2004; 2006), which uses a maximum-entropy approach to model species distributions based on known occurrences and a spatial grid of environmental variables. To model the present distribution of climate suitable for *D. palustris*, we used 19 bioclimatic variables from the WorldClim database (Hijmans et al. 2005) representing average climate from 1950-2000 in 2.5-arc-minute resolution. To guard against overfitting the model, correlations among climate variables were evaluated with ENMTools (Warren et al. 2010), and seven variables were retained for analysis: annual mean temperature, temperature seasonality, minimum temperature of coldest month, mean temperature of wettest quarter, mean temperature of driest quarter, mean temperature of coldest quarter, and annual precipitation.

The spatial-filtering approach of Kramer-Schadt et al. (2013) was used to reduce sampling bias by eliminating redundant collection localities within 100-km radii, after which 67 sites remained for modelling. Random testing was set to 20% by subsampling, the number of replicate analyses was 20, and the maximum number of iterations was 5000. Suitability models were projected onto both the CCSM and MIROC climate reconstructions for the LGM

(Braconnot et al. 2007). Graphical output from MaxEnt was manipulated in ESRI® ArcMap 10.0 (ESRI, Redlands, CA, USA) to identify suitable climate thresholds by using the lowest-presence threshold of Pearson et al. (2007). This approach conservatively estimates areas of potential occurrence because the minimally acceptable climate threshold for projected distributions is equal to the most stringent threshold with no false-negatives (i.e., no known points of occurrence fall outside the range of suitable climate). Regions of potentially suitable climate were identified according to the geomorphic provinces of Fenneman and Johnson (1946).

Chloroplast phylogeography

Sample collection, DNA processing, and sequence editing

We collected bud scales or leaves from plants at least five meters apart in each of 98 populations of *Dirca palustris*, four populations of *Dirca decipiens* (one in Johnson County, Kansas; two in Carroll County, Arkansas; and one in Gasconade County, Missouri), one population of *Dirca mexicana* (Tamaulipas, Mexico), and one population of *Dirca occidentalis* (San Mateo County, California; Table 1; Fig. 1). One plant was sampled from the population of *D. decipiens* in Missouri, and three plants were sampled from each population elsewhere. Leaves were placed immediately into silica gel desiccant. We collected bud scales or young leaves to facilitate DNA extraction when possible; otherwise, mature leaves without evident insect or disease damage were collected. One voucher specimen from each population was deposited at ISC (Thiers 2013; Table 1).

DNA was extracted from samples (Edwards et al. 1991) and purified with an UltraClean® 15 DNA Purification Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) when

initial purity was insufficient for reliable amplification. Quality of DNA was assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). After extraction, DNA was diluted in Tris-EDTA buffer to 100 ng•uL^{-1} and stored in a -20 C freezer.

We chose to amplify the *trnL-trnF, psbD-trnT, rpl32-trnL,* 3'*rps16*-5'*trnK,* and *ndhJ-trnF* noncoding chloroplast regions (Table 2) because preliminary work indicated these regions were readily amplifiable and polymorphic. The chloroplast genome of angiosperms usually is maternally inherited, including for *Daphne*, the only member of the Thymelaeaceae for which inheritance has been confirmed (Zhang et al. 2003). Amplification of each region was performed by polymerase chain reaction in a 25-µL reaction mixture with 12.5 µL GoTaq® Green Master Mix (Promega Corporation, Madison, WI), 10 pmol each of forward and reverse primers (Table 2; Taberlet et al. 1991; Shaw et al. 2007), and 100 ng DNA template. Reaction conditions (adapted from Shaw et al. 2005) were 95 C for 5 min (template denaturation); 30 cycles of 95 C for 1 min (denaturation), 50 C for 1 min (annealing), a ramp of 0.3 C/s to 72 C, and 72 C for 2 min (extension); and 72 C for 5 min (final extension). PCR products were loaded into a TBE gel and subjected to electrophoresis. Amplification products were excised and purified with the UltraClean® 15 DNA Purification Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA).

Purified products were sequenced at the Iowa State University DNA Facility, Ames, IA, with an Applied Biosystems 3730xl DNA Analyzer using BigDye® Terminator cycle sequencing. We edited and aligned sequences with Geneious® 6.1.5 (Biomatters Ltd., Auckland, New Zealand) with manual corrections to the alignment. Sequences with ambiguous base calls were re-sequenced to ensure accuracy. The five regions were

concatenated for further analyses. For each haplotype, a representative sequence from each region was deposited in GenBank (http://www.ncbi.nih.gov/).

Characterization of sequence variation

We used DnaSP v. 5.10.01 (Librado and Rozas 2009) to identify mutational differences among haplotypes and to calculate general measures of genetic diversity. Measurements of sequence data included number of sequences, alignment length, number of substitution sites (base positions), number of insertion/deletion (indel) events and sites, number of haplotypes, haplotype diversity (Hd), nucleotide diversity (π), and average number of nucleotide differences (k).

To investigate further the nature of polymorphism within populations, we amplified and sequenced DNA from an additional seven plants within each of 11 populations across the range of *D. palustris* (Table 1). These included one monomorphic population in each of Florida, Louisiana, Arkansas, West Virginia, and Pennsylvania, and one polymorphic population in each of Tennessee, Missouri, Kentucky, Iowa, Michigan, and Minnesota.

Genetic relationships and divergence time estimates

Genetic variation among haplotypes was depicted with a haplotype network generated from an unrooted neighbor-joining tree constructed with the NEIGHBOR program of PHYLIP (Felsenstein 2005). Neighbor-joining outperforms the statistical parsimony approach implemented in the program TCS (Clement et al. 2000; Salzburger et al. 2011). The neighbor-joining tree was converted into a haplotype network with Fitch lengths (nucleotide differences) by using Haplotype Viewer 1.0 (Salzburger et al. 2011).

We used a Bayesian approach implemented in BEAUti/BEAST v1.7.5 (Drummond et al. 2012) to estimate simultaneously the phylogenetic relationships among individuals and evolutionary time intervals to their most recent common ancestors (TMRCA) for species and lineages. An initial analysis with an uncorrelated, lognormal relaxed clock was run to test for significant substitution rate heterogeneity in the sequence data. Because this analysis failed to justify rejection of a strict clock (distribution of the parameter UCLD.STDEV with mean = 0.418, lower 95% HPD = 0.0, upper 95% HPD = 0.98), we implemented a strict clock to evaluate the plausibility of the mid- to late-Miocene (~10 MYA) as a biogeographic timing event for the isolation of *D. occidentalis* from the remaining taxa of *Dirca* (Graham 1993). Analyses were conducted with a coalescent tree prior, which was chosen to accommodate divergence-time estimates at the intraspecific level (Drummond et al. 2012). The strict molecular clock used a rate of evolution estimated from the data, based on calibration of the tree root to 10 MYA (SD±2.0) according to estimates for the late-Miocene isolation of eastern and western North American flora (Graham 1993). Additional parameters for the analysis included the GTR +I substitution model selected by evaluating the Akaike information criterion in jModeltest (Guindon and Gascuel 2003; Darriba et al. 2012) and a chain length of 20 million with logging of every 1000th tree. We used Tracer v.1.5 (Rambaut and Drummond 2007) to confirm both the convergence of two independent runs and adequate sampling within each run (i.e., effective sample sizes above 200 for all parameters). LogCombiner v.1.7.5 was used to combine analyses with a burn-in of 25% of sampled trees, and TreeAnnotator v.1.7.5 was used to summarize trees.

Population structure

The geographic distribution of haplotypes was visualized by labelling a map of haplotype identities among populations. The latitude of the southernmost (or singular) occurrence of each haplotype was also graphed to depict the latitudes of potential refugia from which haplotypes originated at the onset of postglacial expansion northward. This resulted in relatively conservative predictions of glacial refugia because the possibility of southward expansion was ignored. To test for a correlation between latitude and haplotype richness without the confounding effect of sampling intensity, we ranked populations by latitude and binned them into groups of 14. These groups of equal size were used for regression to test for spatial trends in haplotype richness, which is presumed to be greatest near glacial refugia. Potential locations of refugia, based on haplotpic evidence, were identified according to the geomorphic provinces of Fenneman and Johnson (1946).

Phylogeographic structure in the variation of haplotypes was assessed generally by a Mantel test (Mantel 1967) and by spatial autocorrelation (Miller 2005), with two specific hypotheses tested by analysis of molecular variance (AMOVA; Excoffier et al. 1992). Although AMOVA is a powerful approach for identifying geographic structure in genetic variation, the approach requires users to identify *a priori* the groupings of individuals to evaluate. Therefore, we also implemented Monmonier's algorithm (Monmonier 1973; Miller 2005), which can detect genetic structure without *a priori* selection of population groupings.

The Mantel test in Alleles in Space (AIS; Miller 2005) was used to analyze for evidence of isolation-by-distance (i.e., limited gene flow) among populations. This test evaluated the correlation between pairwise genetic mismatch distance and geographic distance to test observations against a null model of no structure. The test for spatial autocorrelation

was conducted by using AIS, which calculated the average pairwise genetic distance among individuals within each of 10 equal distance classes. We used 1000 permutations and UTM grid coordinates for both analyses (Miller 2005). We conducted AMOVA in Arlequin 3.5 (Excoffier and Lischer, 2010) with 999 permutations to measure variation among populations. This approach is based on the identification of pairwise genetic distances among all individuals sampled and the partitioning of total diversity into within- and among-population components. Fixation indices (ϕ-statistics) calculated for each hierarchical level were tested by comparing the variance at each level to a null model with random distribution of variance. We tested the roles of two plausible barriers to east-west seed dispersal: the combined influence of the Appalachian Mountains and Apalachicola River, and the Mississippi River.

Monmonier's algorithm in AIS (Miller 2005) was implemented by using residual genetic distances to detect putative barriers to seed dispersal among populations. This algorithm evaluates the spatial structure of genetic variation and identifies barriers to gene flow along a population-connectivity network (Monmonier 1973). We evaluated the outcomes of directing the AIS software to select one, three, six, and nine barriers, resulting in a depiction of the order in which the algorithm identified putative barriers.

Results

Niche modelling and LGM distribution

Ecological niche modelling revealed a broad distribution of suitable climate during the LGM, including several mid-latitudinal regions (Fig. 2). The 67 populations selected for ENM (Fig. 2A) produced a model with good predictive ability (AUC = 0.83). Suitable climate was predicted outside the known distribution of *D. palustris*, and in northeastern Mexico where *D.*

mexicana occurs (Fig. 2B). Although both LGM climate reconstructions indicated a belt of climate highly suitable for *D. palustris* in the southeastern U.S., the models differed. The CCSM climate reconstruction produced a more southerly distribution of suitable climate and fewer opportunities for persistence of *D. palustris* in mid-latitudinal refugia (Fig. 2C), whereas the MIROC climate reconstruction led to a larger and more northerly belt of suitable habitat (Fig. 2D). The MIROC data indicated a much more extensive presence of likely suitable habitat in mid-latitudinal refugia, including climatically suitable sites in northern Arkansas (Ozark Plateaus Province), West Virginia and eastern portions of Tennessee (Appalachian Plateaus Province), and the Piedmont and Coastal Plain Provinces as far north as Maryland.

Characterization of sequence variation

Sequence variation was detected at the genus level, along with considerable diversity within *D. palustris* (Table 3). Among all samples we identified 39 distinct haplotypes, 35 of which belonged to *D. palustris.* No intraspecific haplotypic variation was found within *D. mexicana* or *D. occidentalis,* but we found two haplotypes in *D. decipiens* (Table 1). Although the putative number of mutations was much higher with the inclusion of all samples (Table 3), those from *D. palustris* alone showed 47 putative mutations (substitutions + indels). Other measures of molecular diversity are provided to characterize the sequence data (Table 3).

Among the 11 populations from which we sequenced 10 individuals, all populations that were monomorphic when three plants were initially sequenced remained monomorphic when 10 plants were sequenced. Of the six polymorphic populations from which we sequenced additional plants, we identified in two of them a third haplotype that was also present in nearby populations. No additional haplotypes were identified in the four remaining

populations. Because additional sequencing revealed new haplotypes in only two of the 11 populations, we maintained a sample of three individuals per population to assess variation in *D. palustris*.

Genetic relationships and divergence-time estimates

We identified evidence for deep genetic divergence among *Dirca* species and considerable divergence within *D. palustris*. The haplotype network (Fig. 3A) indicated that *D. occidentalis* was genetically distinct, with 235 nucleotides (including indels) differing between it and *D. mexicana*. *Dirca mexicana* and *D. decipiens* were genetically similar, with 14 nucleotide differences corresponding to several mutational differences. Within *D. palustris*, major lineages often had multiple mutational steps, both among and within lineages. Twenty-three population-specific haplotypes also were identified (labelled white circles, Fig. 3A). Bayesian analysis provided strong evidence for monophyly of *Dirca palustris* and moderate evidence for the monophyly of the *D. decipiens* and *D. mexicana* clade (Fig. 3B). The Bayesian tree had moderate to high posterior probabilities (≥ 0.70) for 13 distinct clades within *D. palustris*, although several internal nodes were not well supported, indicating insufficient resolution of chloroplast mutations to resolve the phylogenetic relationships among clades. The TMRCA for all extant *Dirca* was calculated by Bayesian analysis to be 9.1 MYA, with a corresponding rate of sequence evolution estimated at 1.06×10^{-3} substitutions•site^{-1}•year^{-1}. The TMRCA between the *D. mexicana/D. decipiens* lineage and the *D. palustris* lineage (Fig. 3B) was ~1.0 MYA, and extant *D. palustris* shared a MRCA living ~620 thousand years ago (KYA). Furthermore, Bayesian analysis demonstrated strong support for several clades within *D. palustris* with estimated TMRCA of 39 KYA to 217

KYA (Fig. 3B). The estimated TMRCA between *D. mexicana* and *D. decipiens* was ~500 KYA, suggesting a time since divergence between the two species that is less than that among some lineages within *D. palustris.*

Population structure

Various haplotypes were widely scattered across the range of *D. palustris*, and the distribution of individuals revealed little evidence for geographic structuring by major clades (Fig. 4). The most abundant haplotype (S-11) was restricted primarily to the western part of the range. Many haplotypes were identified in mid-latitudinal populations. Several distinct haplotypes were identified in the Gulf Coastal Region, two of which (S-1 and S-6) occurred in several populations in the southern U.S. (≤35 °N) but nowhere else, and two of which (P-5 and P-21) occurred in one population each in Mississippi and Alabama. We identified eight additional population-specific haplotypes within the Atlantic Coastal Plain and Piedmont Provinces. Several additional rare or population-specific haplotypes were identified at northern latitudes in the Great Lakes Region and northern Minnesota. The southernmost (or singular) occurrences of most haplotypes were between 35-40°N, and the greatest haplotype richness was observed in these middle latitudes due to the high genetic diversity in putative mid-latitudinal refugia of the Ozark Plateaus Province, the Interior Lowland Plateaus Province, and the southern portion of the Appalachian Plateaus Province (Figs. 4, 5).

Analyses of population structure indicated spatial structure among populations and regions. A Mantel test showed evidence for isolation-by-distance, but with a low correlation coefficient ($r^2 = 0.08$; $P = 0.001$). Analysis of spatial autocorrelation showed that pairwise genetic distances were less than average at shorter distances and greater than average at longer

distances, with the exception of the greatest distance class, for which genetic distances were not different from the average (Fig. 6). AMOVA showed that a significant portion of total genetic variation was among populations (Table 4). Between the two scenarios of geographic differentiation considered, the greater between-group variation (11.2%) was for populations to the east and the west of the Appalachian Mountain/Apalachicola River barrier. There was no evidence that the Mississippi River posed a barrier to gene flow (P=0.406; Table 4).

Analysis by Monmonier's algorithm first identified a genetic discontinuity in the southwestern portion of the distribution of *D. palustris* (Fig. 7). Allowing the AIS software to place two more barriers led to identification of two regions of genetic discontinuity in the putative mid-latitudinal refugia of the Appalachian Plateaus Province. Instructed to identify six putative barriers, the software further separated the southern portion of the range from mid- and upper-latitudes, and identified an area of genetic discontinuity in the northwestern corner of the species distribution. With nine barriers, there were genetic discontinuities identified along the Appalachian Mountain range, but much of the Great Lakes Region and upper midwestern U.S. remained genetically contiguous with putative mid-latitudinal refugia at 35-40 °N (Fig. 7).

Discussion

Existence of mid-latitudinal refugia of *D. palustris* during the LGM is plausible based on climatic modelling (Fig. 2), and genetic evidence supports the persistence of such refugia during the LGM at ~35-40 °N (Figs. 4, 5, 7). Furthermore, late Pleistocene refugia along the Gulf Coast are unlikely sources of propagules responsible for the recent, interglacial migration of *D. palustris* to the north (Fig. 4). Six populations of white-pubescent plants in the

southeastern U.S., where the species is rare, share a distinct haplotype and may warrant attention by conservationists. Finally, *D. palustris* is genetically diverse with deep splits among chloroplast lineages (Fig. 3), indicating a complex phylogeographic history not readily described as a product of the LGM alone.

Evaluation of the plausibility of mid-latitudinal refugia by ENM has become an important component of phylogeographic studies (Rissler and Apodaca 2007; Morris et al. 2010; Chen et al. 2012). ENM provides a biologically constrained approach for testing hypotheses related to geographic histories of species that is independent of molecular techniques (Waltari et al. 2007). Thus, the convergence of ENM and molecular evidence constitutes compelling support for the hypothesis of mid-latitudinal LGM refugia. In this case, the ENM established the plausibility of mid-latitudinal refugia located in regions that were previously indicated likely for various temperate forest species by other methods (Jackson et al. 2000; Rowe et al. 2004; McLachlan et al. 2005), including the Ozark Plateaus Province, the Interior Lowland Plateaus Province, and the southern portion of the Appalachian Plateaus Province (Fenneman and Johnson 1946).

An additional consideration concerning the distribution of temperate species during the LGM is evidence for microrefugia, small pockets of suitable climate and habitat in regions of generally unsuitable climate. Such refugia were likely too small and scattered to be identified by ENM (Dobrowski 2011), thereby rendering plausible the notion that species like *D. palustris* could have persisted even further north during the LGM than indicated by ENM. Indeed, there are reasons to suspect traditional LGM climate hypotheses based on pollen assemblages mistook competitive effects of low CO_2 at higher latitudes for colder climate

(Loehle 2007). Consequently, many temperate species may not have retreated far south of the glacial ice sheet.

Several aspects of our results indicate that *D. palustris* may have maintained an extensive regional population relatively close to the Laurentide ice sheet during the LGM. The high diversity of haplotypes at mid-latitudes and the southernmost occurrences of many haplotypes at middle to high latitudes (Figs. 4, 5) suggest some refugia existed within 500 kilometers from the continental ice sheet (35-40 °N), thereby facilitating relatively rapid expansion into previously unsuitable regions. McLachlan et al. (2005) made similar conclusions for *Fagus grandifolia* Ehrh. and *Acer rubrum* L.*,* trees common in forests occupied by *D. palustris* where the species are sympatric. Further evidence for multiple, mid-latitudinal refugia is the presence of geographic breaks throughout those latitudes (Fig. 7). Moreover, the presence of an unusually high number of rare or endemic taxa in the Ozark Plateaus Province, including *D. decipiens* (Fig. 4), constitutes strong evidence that the region acted as a haven for relictual populations of once-broader taxa during the LGM (The Nature Conservancy 2003; Tribsch and Schönswetter 2003). Similarly, the high degree of biological endemism for a variety of temperate taxa in the southern portion of the Appalachian Plateaus Province (Braun 1951) casts serious doubt on the traditional hypothesis of primarily southern refugia for temperate species (Loehle 2007). The presence of many temperate endemics just south of the LGM ice margin, with few in previously glaciated regions, indicates that wholesale migration of temperate flora during the LGM is unlikely, and that a heterogeneous assortment of boreal and temperate taxa and tundra may have persisted near the ice margin (Loehle 2007). Finally, the absence of multiple southern haplotypes from middle to upper latitudes suggests relatively limited northward dispersal from the Gulf Coastal Region (Fig. 4). Collectively, the results of

this study are consistent with an accumulating body of genetic evidence that northward range expansion of temperate forest species occurred not from low-latitudinal refugia as previously hypothesized, but from more interior refugia or microrefugia close to the glacial margin (McLachlan et al. 2005; Soltis et al. 2006; Gonzales et al. 2008).

The relatively high haplotypic diversity in the upper Great Lakes Region is intriguing (Fig. 4). Two possibilities might account for this heterogeneity in the upper Great Lakes Region, including diffusive migration from genetically rich refugia south of the LGM ice margin, or repeated long-distance dispersal. The mixing of many distinct haplotypes suggests repeated long-distance dispersal, whereby genetic variation from more southern sites was sampled and dispersed to produce a mosaic of genetic diversity in the upper midwestern U.S. However, patterns of genetic diversity expected by differing means of dispersal are complex and not readily assessed (Bialozyt et al. 2006). Because of its relative absence of obvious dispersal mechanisms, *D. palustris* may have diffused slowly to the north during the present interglacial period. The presence of mid-latitudinal refugia near the glacial margin supports the plausibility of slow diffusion, with migration as low as tens of metres per year accounting for the current northward distribution of the species. However, such a rate of routine dispersal is higher than our observations of *D. palustris* would suggest is likely, because fruits seem unappealing to birds and generally fall to the forest floor and incorporate into the leaf litter (Peterson and Graves 2011). We speculate that stratified dispersal to the north, characterized by both diffusion and occasional long-distance dispersal by active vectors, might account for some of the genetic heterogeneity of the upper Great Lakes Region (Bialozyt et al. 2006).

A prime candidate as an agent for long-distance dispersal is the now-extinct migratory passenger pigeon (*Ectopistes migratorius* L.), once the most common bird in North America

(Schorger 1955). It routinely migrated from southern hardwood forests in the spring, eventually aggregating into massive breeding colonies throughout the Great Lakes Region (Ellsworth and McComb 2003). These birds were generalists that stripped foraging sites of nuts, fruits, and insects before seeking new feeding grounds (Schorger 1955). Passenger pigeons, which had the capacity for rapid flight (~100 km•hour^{-1}) and delayed digestion, routinely regurgitated low-quality food in favor of more palatable or nutritious sustenance, rendering plausible the hypothesis that this bird aided dispersal of many temperate forest species to previously glaciated parts of eastern North America (Schorger 1955; Webb 1986).

Our phylogenetic evidence indicated both deep divergence among maternal lineages (Fig. 3) and a widespread, weakly structured distribution of haplogroups across the landscape (Figs. 4, 6; Table 4; Mantel test). This presence of deep maternal diversification, coupled with a lack of geographic fidelity of lineages by region, suggests a complex Pleistocene history of isolation and diversification during repeated glacial maxima with expansion and subsequent admixture of maternal haplotypes during interglacial periods (Moncrief et al. 2012; Chen et al. 2013; Hewitt 2000). Patterns observed for *D. palustris* differ markedly from the high geographic fidelity of major lineages in taxa subjected to long-term isolation in separate Pleistocene refugia during repeated climate oscillations (Hewitt 2004). However, the white-pubescent plants sharing a distinct haplotype in the Southeast (S-1; Fig. 3; Fig. 4) are sufficiently restricted in distribution that their isolation from other populations for thousands of years is plausible. An assessment of the nuclear genomes of plants in those populations is warranted because diversity of nuclear genomes cannot be inferred from the sort of deep diversification of maternal lineages that we have shown in *D. palustris* (Soltis and Kuzoff 1995).

Although strong taxonomic conclusions should not be made at the intraspecific level based on chloroplast DNA, our data shed light on the evolution and biogeography of the genus *Dirca.* Our analyses with a strict molecular clock calibrated to a late-Miocene node age (~10 MYA) for all *Dirca* (Fig. 3) indicated a rate of chloroplast mutation (1.06×10^{-9} substitutions•site^{-1}•year^{-1}) close to that estimated by others for chloroplast evolution (1.1×10^{-9} to 1.3×10^{-10}; Curtis and Clegg 1984; Wilson et al. 1990). Therefore, we are confident that the generally recognized isolation of North America's eastern and western flora following the uplift of the Rocky Mountains and mid-continental drying and cooling during the mid- to late-Miocene (Graham 1993; Xiang et al. 2000) accounts for the divergence between *D. occidentalis* and eastern *Dirca.* This ancient divergence, recognized by Nesom and Mayfield (1995), Schrader and Graves (2004), and Floden et al. (2009), resulted from disjunction of *Dirca* following widespread distribution during the early Miocene.

Later diversification within eastern *Dirca* took place exclusively during Pleistocene climate oscillations that interrupted floristic continuity between eastern North America and Mexico established during the middle to late Miocene (Graham 1993; Nesom and Mayfield 1995). Our analysis places vicariance between the *D. mexicana/D. decipiens* lineage and the *D. palustris* lineage (~1.1 MYA) in the Calabrian stage, during which the mid-Pleistocene transition to more frequent glaciations of greater amplitude occurred (ca. 0.9 MYA; Ruddiman et al. 1989). We suggest that the *D. mexicana/D. decipiens* lineage persisted during this transitional period in a refugium in Mexico, with subsequent divergence into *D. decipiens* and *D. mexicana* occurring during the Ionian stage of the Middle Pleistocene (~500 KYA). It is unclear how and when *D. decipiens* returned to mid-latitudes and came to occupy the western limit of the range of *D. palustris*, although it probably happened during one of the late-

Pleistocene interglacials, after which the lineage eventually survived the LGM as an endemic of the Ozark Plateaus Province. Overall, our divergence estimates based on sequence data are consistent with interpretations by Nesom and Mayfield (1995) concerning the disjunction between *Dirca* of the eastern deciduous forests and the mountains of northeastern Mexico. The alternative hypothesis proposed by Axelrod (1975) of simultaneous, mid-Oligocene fragmentation of a widespread flora extending from the western United States through Mexico to eastern North America is not supported by our results.

We have demonstrated here the plausibility of mid-latitudinal refugia for *D. palustris* during the LGM, and our genetic evidence suggests that the species occupied habitats within several hundred km of the Laurentide ice sheet. Accumulating genetic evidence for the persistence of such refugia, coupled with occasional long-distance seed dispersal, resolves this instance of Reid's paradox. Our results leave uncertain the degree to which migration of *D. palustris*, with its patchy distribution and no evidence for contemporary seed dispersal beyond the immediate vicinities of maternal parents, will keep pace with future migration of suitable climate. An exclusive process of slow diffusion from mid-latitudinal refugia would suggest a limited capacity for distributions of temperate plants like *D. palustris* to shift with rapidly changing climates. Likewise, if ecological interactions that once favored long-distance dispersal are diminished or absent in contemporary landscapes, as is certainly the case for numerous taxa once primarily dispersed by the passenger pigeon, then dispersal limitation could play a significant role in future range contractions and extinctions.

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Table 1. Locations, voucher specimens, and haplotypes of *Dirca palustris* sampled for chloroplast sequencing. One voucher is deposited in ISC (Ames, IA) from each population in which specimens were collected. Names of *D. palustris* haplotypes correspond to labels in Fig. 3. Either three or 10 individuals were sequenced from each population. Asterisks preceding county names denote populations from which we sequenced 10 individuals. In the two populations with three haplotypes, the third haplotype was not identified until the additional seven plants were sequenced.

Species	County	State	Latitude	Longitude	Voucher	Haplotype
Dirca occidentalis						
	San Mateo	California	37.407503	-122.236054	--	--
Dirca mexicana						
	n/a	Tamaulipas	23.985833	-99.476944	2011-100A	--
Dirca decipiens						
	Carroll	Arkansas	36.169167	-93.544759	2011-21A	D-1
	Carroll	Arkansas	36.465955	-93.760285	2011-19A	D-1
	Gasconade	Missouri	38.514333	-91.609058	Floden 984	D-2
	Johnson	Kansas	38.795838	-94.691634	2011-18A	D-1

Table 1 continued

Dirca palustris

Clarke	Alabama	31.484766	-87.851068	--	S-11
Colbert	Alabama	34.622508	-87.794323	2011-51A	S-8
Henry	Alabama	31.360830	-85.111100	--	S-1
Marshall	Alabama	34.475076	-86.059406	2011-50A	S-9
Monroe	Alabama	31.546393	-87.515631	2011-24A	S-11
Tuscaloosa	Alabama	33.280556	-87.406111	2011-49A	P-5
Wilcox	Alabama	31.818367	-87.384789	--	S-11
Wilcox	Alabama	31.906589	-87.380930	2011-48A	S-1
Winston	Alabama	34.283010	-87.395580	2011-53A	S-2, S-11
*Carroll	Arkansas	36.175920	-93.560042	2011-22A	S-7
Garland	Arkansas	34.523745	-93.394406	2011-47A	S-11
Howard	Arkansas	34.321154	-94.228551	2011-46A	S-9
Madison	Arkansas	35.895430	-93.582230	2011-20A	S-7, S-12
Madison	Arkansas	36.042419	-93.488781	--	S-7

Table 1 continued

Madison	Arkansas	36.074223	-93.488696	--	S-2
Newton	Arkansas	36.016789	-93.350444	2011-13A	P-8
Newton	Arkansas	36.028100	-93.173925	2011-16A	S-9
Stone	Arkansas	35.996063	-92.210655	2011-23A	S-11
Gadsen	Florida	30.626095	-84.902344	2011-56A	S-1
*Liberty	Florida	30.590637	-84.935303	2011-55A	S-1
Clay	Georgia	31.616136	-85.049303	2011-54A	S-6
Decatur	Georgia	30.766582	-84.644959	--	S-1
Early	Georgia	31.353875	-85.064258	--	S-6
Pope	Illinois	37.527154	-88.648682	--	S-9
Stephenson	Illinois	42.442936	-89.759116	--	S-2, S-11
Parke	Indiana	39.887444	-87.199419	2011-72A	S-11
*Boone	Iowa	41.992924	-93.886753	2011-32A	S-2, S-11
Clayton	Iowa	42.678397	-91.398010	2011-42A	S-11
Clayton	Iowa	42.813584	-91.340343	2011-41A	S-2, S-11

Table 1 continued

Webster	Iowa	42.420193	-94.102235	2011-31A	S-11
Adair	Kentucky	37.010886	-85.498683	2011-65A	S-4, S-11
Breathitt	Kentucky	37.458740	-83.164010	2011-64A	S-12
*Breckinridge	Kentucky	37.817280	-86.292909	2011-29A	S-3, S-10
Carter	Kentucky	38.357575	-83.111572	2011-67A	P-6
Franklin	Kentucky	38.287377	-84.860158	2011-28A	S-10
Hardin	Kentucky	37.716313	-85.74758	2011-30A	S-4
Jackson	Kentucky	37.428524	-83.921127	2011-63A	S-2
Laurel	Kentucky	37.174197	-84.280901	2011-62A	S-9
*Catahoulah	Louisiana	31.845223	-91.952627	2011-45A	S-11
Aroostook	Maine	45.931283	-68.320000	2011-75A	P-7
Berkshire	Massachusetts	42.672200	-73.252883	2011-78A	S-11
Hampshire	Massachusetts	42.309917	-72.524217	2011-77A	S-9
Alger	Michigan	46.381584	-87.077062	2011-82A	S-11
Benzie	Michigan	44.769155	-86.054513	2011-103A	S-11

Table 1 continued

*Berrien	Michigan	41.910382	-86.602865	2011-101A	S-11, P-20
Cheboygan	Michigan	45.485141	-84.685864	2011-104A	S-4
Gogebic	Michigan	46.263780	-89.257430	2011-85A	S-9
Iron	Michigan	46.094646	-88.429657	2011-84A	S-9
Oakland	Michigan	42.650139	-83.558865	2011-102A	S-8, P-14
Clearwater	Minnesota	47.185761	-95.186577	2011-94A	P-11
Nicollet	Minnesota	44.26686	-94.041325	2011-90A	S-10
Olmsted	Minnesota	44.101436	-92.135822	2011-35A	S-11
Polk	Minnesota	47.61236	-96.050198	2011-93A	S-7, S-11
Ramsey	Minnesota	44.942142	-93.197236	2011-33A	S-11
*St. Louis	Minnesota	47.214236	-93.055252	2011-98A	S-7, P-10
Todd	Minnesota	45.83063	-94.664244	2011-92A	S-2
Wayne	Mississippi	31.829662	-88.529470	--	P-21
Barry	Missouri	36.579048	-93.835477	2011-44A	S-2
Boone	Missouri	38.758913	-92.204664	2011-3A	S-3

Table 1 continued

Grundy	Missouri	40.118802	-93.682319	2011-1A	S-11
Howell	Missouri	36.917770	-92.083330	2011-4A	P-17
*Madison	Missouri	37.379804	-90.498671	2011-15A	S-5, S-9; S-11
Maries	Missouri	38.144548	-91.801243	2011-2A	S-5, S-11
Oregon	Missouri	36.787188	-91.345160	2011-27A	S-5
Wayne	Missouri	37.260802	-90.504502	2011-14A	S-10, P-18
Wright	Missouri	37.093629	-92.679548	2011-5A	S-11
York	New Brunswick	45.992183	-66.867933	2011-73A	P-4
Strafford	New Hampshire	43.229583	-71.071383	2011-79A	S-7
Westchester	New York	41.210254	-73.561049	2011-70A	P-3
Caswell	North Carolina	36.344509	-79.206383	2011-68A	P-16
Cleveland	North Carolina	35.200961	-81.666277	2011-61A	S-1
Cavalier	North Dakota	48.964722	-98.100278	2011-40A	S-7
Hants	Nova Scotia	45.037833	-63.46845	2011-74A	S-9
Butler	Ohio	39.552765	-84.732571	2011-71A	S-4, P-15

Table 1 continued

Forest	Pennsylvania	41.536173	-79.436436	2011-88A	S-7
*Potter	Pennsylvania	41.485100	-77.728600	2011-87A	S-9
Aiken	South Carolina	33.542327	-81.995834	2011-60A	S-9
Cherokee	South Carolina	35.026807	-81.490370	--	P-2
Fairfield	South Carolina	34.429740	-81.113965	2011-58A	P-1
Fairfield	South Carolina	34.456536	-81.394315	2011-59A	S-9
Fentress	Tennessee	36.524430	-84.888570	--	P-12
Hickman	Tennessee	35.874107	-87.449359	2011-12A	S-2
Lawrence	Tennessee	35.090217	-87.471530	2011-52A	S-2, S-9
Polk	Tennessee	35.120874	-84.563205	Floden/Hart 1973	S-10
Polk	Tennessee	35.181394	-84.441701	2011-9A	S-7
Scott	Tennessee	36.480242	-84.662962	2011-6A	S-8
*Tazwell	Tennessee	36.402183	-83.456783	--	P-13, P-19; S-10
Washington	Vermont	44.285700	-72.437500	2011-76A	P-23
Page	Virginia	38.626929	-78.341875	2011-69A	P-9

Table 1 continued

*Summers	West Virginia	37.719659	-80.890493	2011-66A	S-4
Door	Wisconsin	45.068982	-87.145228	2011-80A	S-11
Door	Wisconsin	45.141646	-87.194302	2011-81A	S-11
Douglas	Wisconsin	46.620762	-91.608392	2011-97A	S-11, P-22
Grant	Wisconsin	43.014849	-90.758853	2011-39A	S-11
Marathon	Wisconsin	44.902855	-90.221892	2011-36A	S-3, S-11
Taylor	Wisconsin	45.121018	-90.61213	2011-37A	S-10
Vernon	Wisconsin	43.690068	-90.795502	2011-34A	S-11
Wood	Wisconsin	44.530772	-90.071980	2011-38A	S-11, S-7

Table 2. Noncoding chloroplast regions selected to study the phylogeography of *Dirca palustris*. Primers TabC and TabF are from Taberlet et al. (1991). All other primers are from Shaw et al. (2007). Primer names followed by an asterisk were also used in sequencing reactions. Single primers were used to sequence regions 3'*rps16*-5'*trnK* and *ndhJ-trnF* because they were sufficient to sequence most of each region.

Region	Primer	Primer sequence (5' – 3')
trnL-trnF	TabC *	CGAAATCGGTAGACGCTACG
	TabF *	ATTTGAACTGGTGACACGAG
ndhJ-trnF	ndhJ	ATGCCYGAAAGTTGGATAGG
	TabE *	GGTTCAAGTCCCTCTATCCC
psbD-trnT	psbD *	CTCCGTARCCAGTCATCCATA
	$trnT^{(GGU)}$-R *	CCCTTTTAACTCAGTGGTAG
rpl32-trnL	$trnL^{(UAG)}$ *	CTGCTTCCTAAGAGCAGCGT
	rpL32-F *	CAGTTCCAAAAAAACGTACTTC
3'*rps16*-5'*trnK*	rpS16x2F2 *	AAAGTGGGTTTTTATGATCC
	$trnK^{(UUU)}$	TTAAAAGCCGAGTACTCTACC

Table 3. Summary statistics based on sequence data from *Dirca palustris*; *D. palustris, D. mexicana,* and *D. decipiens*; and all four species of *Dirca.*

Parameter	*D. palustris*	All except *D. occidentalis*	All four species
No. sequences	294	307	310
Alignment length	4576	4586	4615
No. substitution sites	30	38	105
No. indel events (indel sites)	17 (97)	29 (112)	36 (272)
No. haplotypes (substitutions + indels)	35	38	39
Haplotype diversity (Hd)	0.893	0.901	0.9030
Nucleotide diversity (π)	0.00062	0.00070	0.00102
Average no. nucleotide differences (k)	2.788	3.134	4.447

Table 4. Analysis of molecular variance for chloroplast haplotypes of *Dirca* in eastern North America.

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	Fixation index	*P*-value
Populations						
Among populations	97	1397.54	4.65	86.03	F_{ST} 0.86	<0.001
Within populations	190	143.33	0.75	13.97		
East vs. west of Appalachian/ Apalachicola Barrier						
Among regions	1	84.36	0.63	11.22	F_{CT} 0.11	<0.001
Among populations within regions	96	1257.59	4.20	75.26	F_{SC} 0.85	<0.001
Within populations	190	143.33	0.75	13.51	F_{ST} 0.86	<0.001
East vs. west of Mississippi River						
Among regions	1	12.09	-0.02	-0.30	F_{CT} 0.00	0.406
Among populations within regions	96	1329.86	4.46	85.79	F_{SC} 0.86	<0.001
Within populations	190	143.33	0.75	14.52	F_{ST} 0.85	<0.001

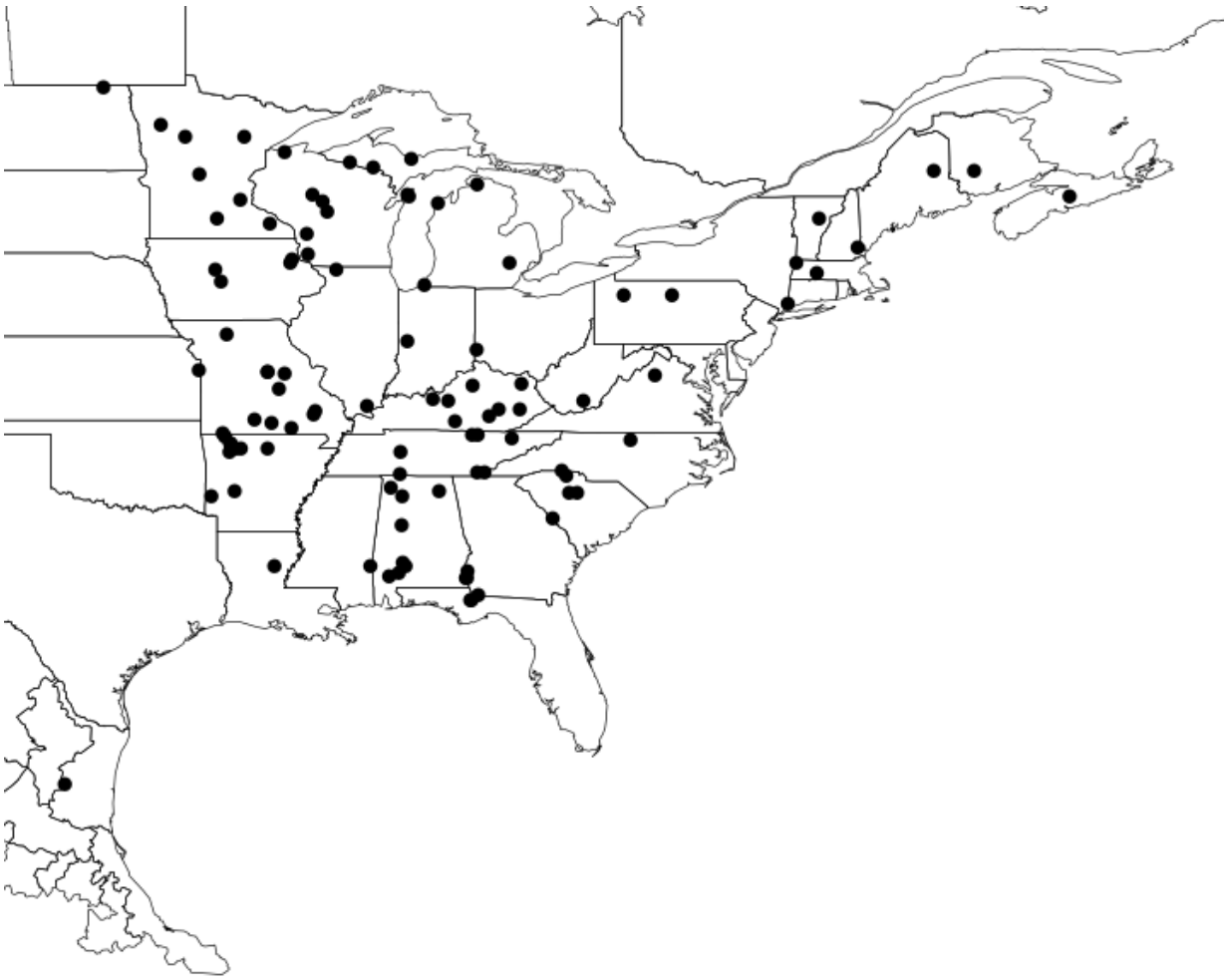

Fig. 1.

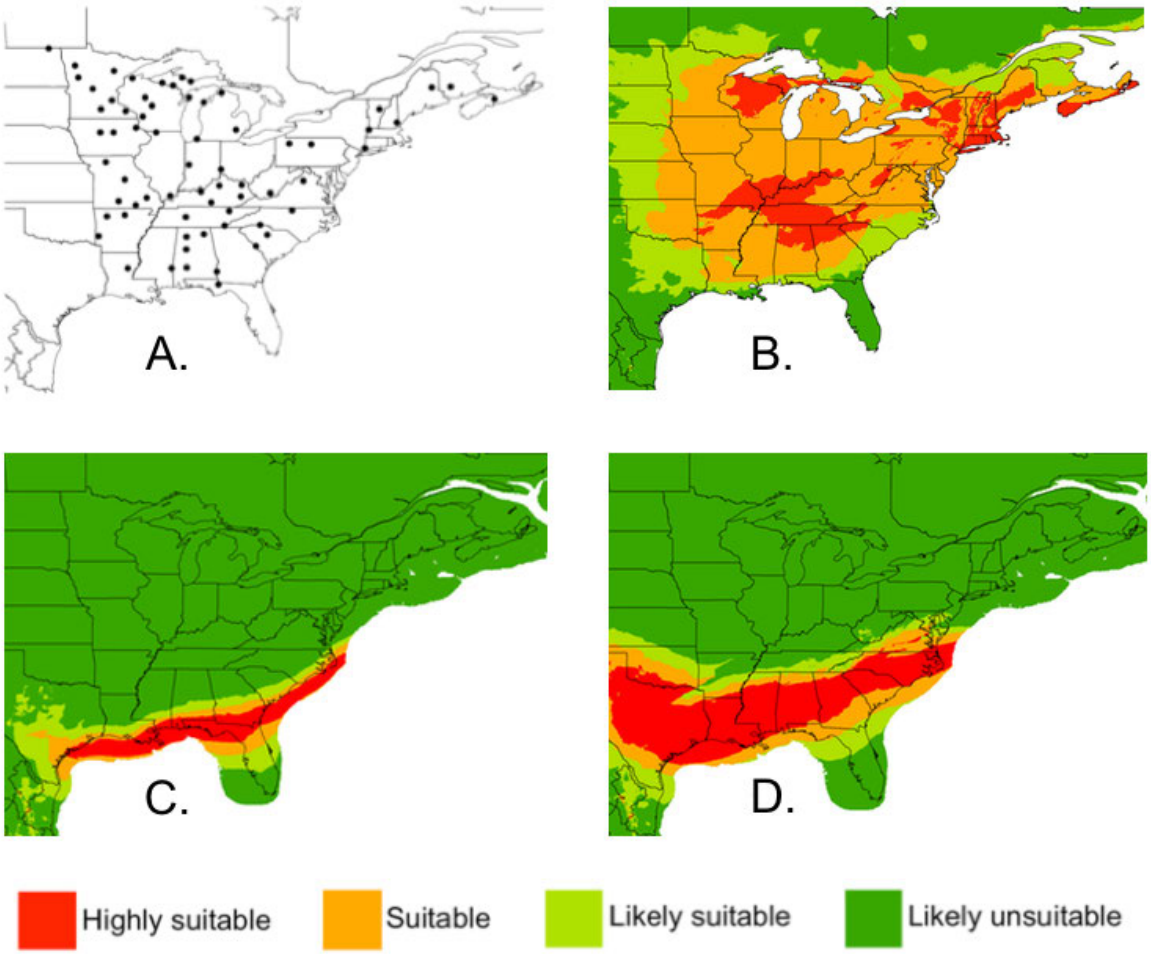
A.
B.
C.
D.
Highly suitable
Suitable
Likely suitable
Likely unsuitable

Fig. 2.

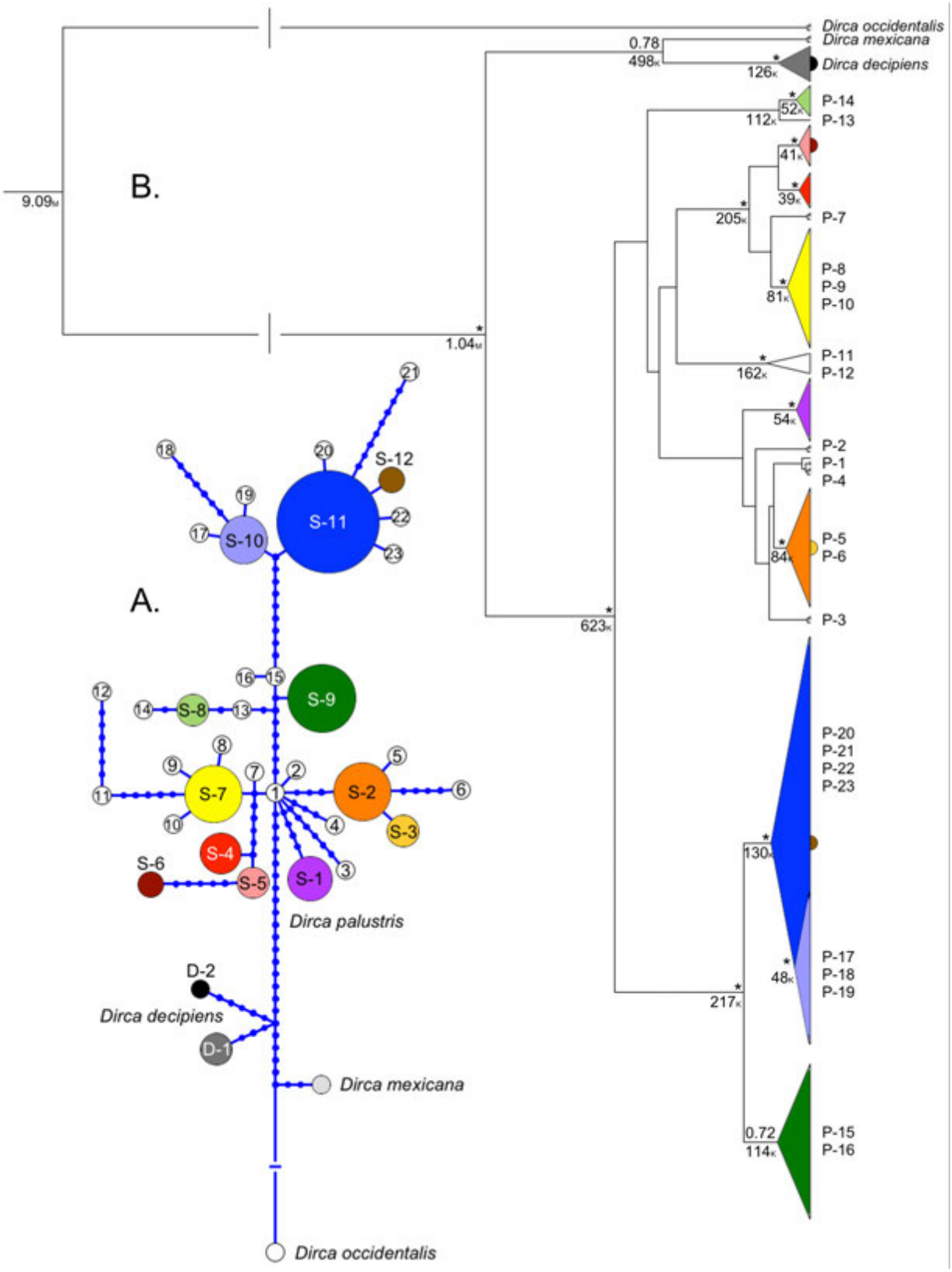
B.
9.09M
1.04M
Dirca occidentalis
Dirca mexicana
Dirca decipiens
0.78
498K
126K
52K
112K
P-14
P-13
41K
39K
205K
P-7
81K
P-8
P-9
P-10
162K
P-11
P-12
54K
P-2
P-1
P-4
84K
P-5
P-6
P-3
623K
A.
S-12
S-11
S-10
S-9
S-8
S-7
S-2
S-3
S-4
S-5
S-1
S-6
Dirca palustris
D-2
Dirca decipiens
D-1
Dirca mexicana
Dirca occidentalis
P-20
P-21
P-22
P-23
130K
48K
P-17
P-18
P-19
217K
0.72
114K
P-15
P-16

Fig. 3.

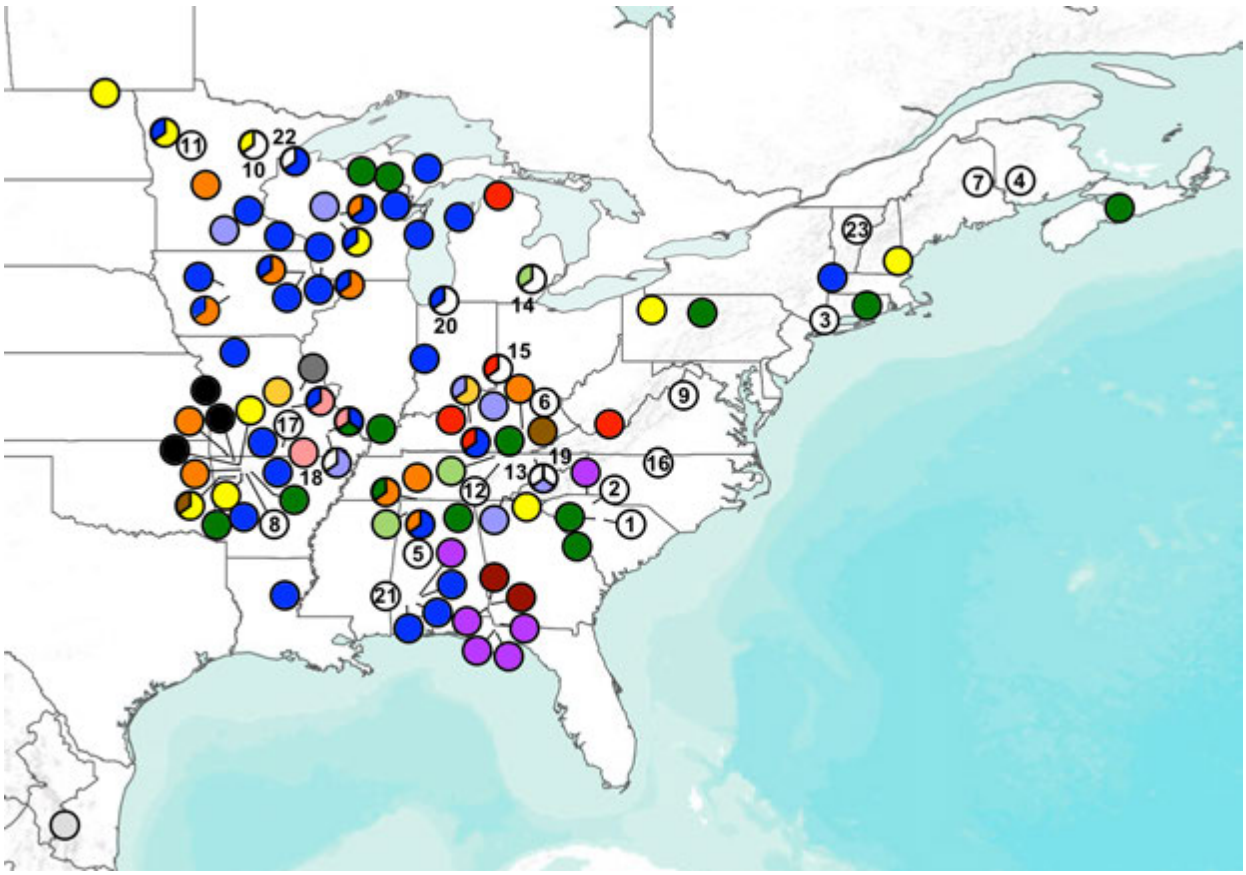

Fig. 4.

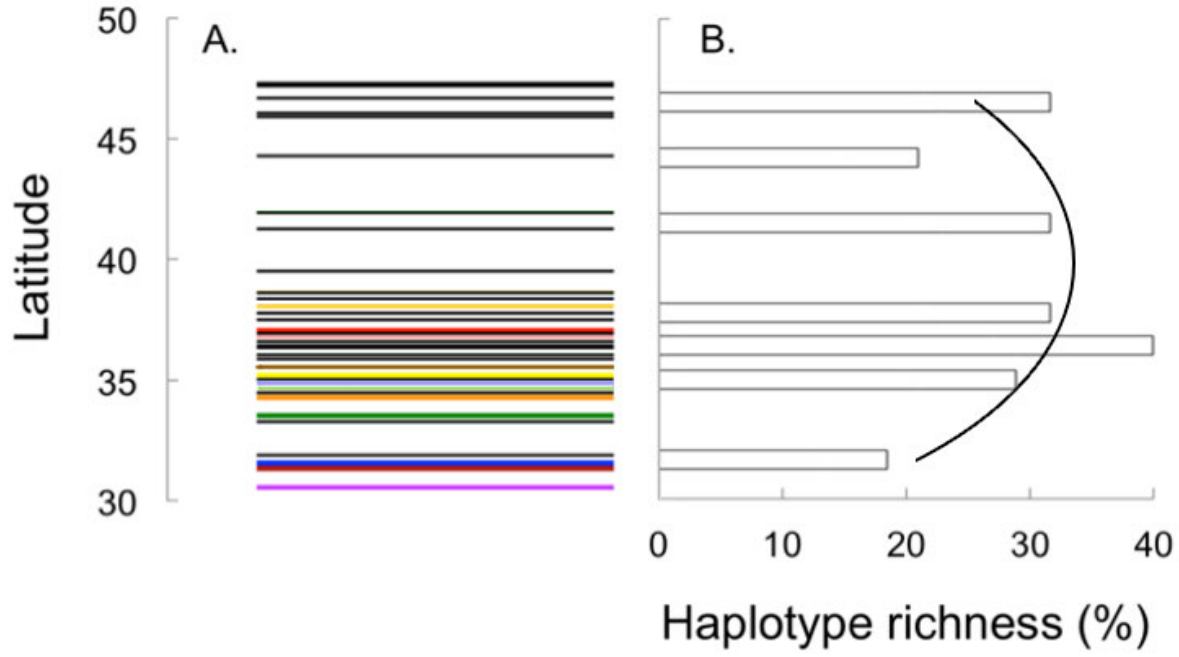
A.
B.
Latitude
50
45
40
35
30
0
10
20
30
40
Haplotype richness (%)

Fig. 5.

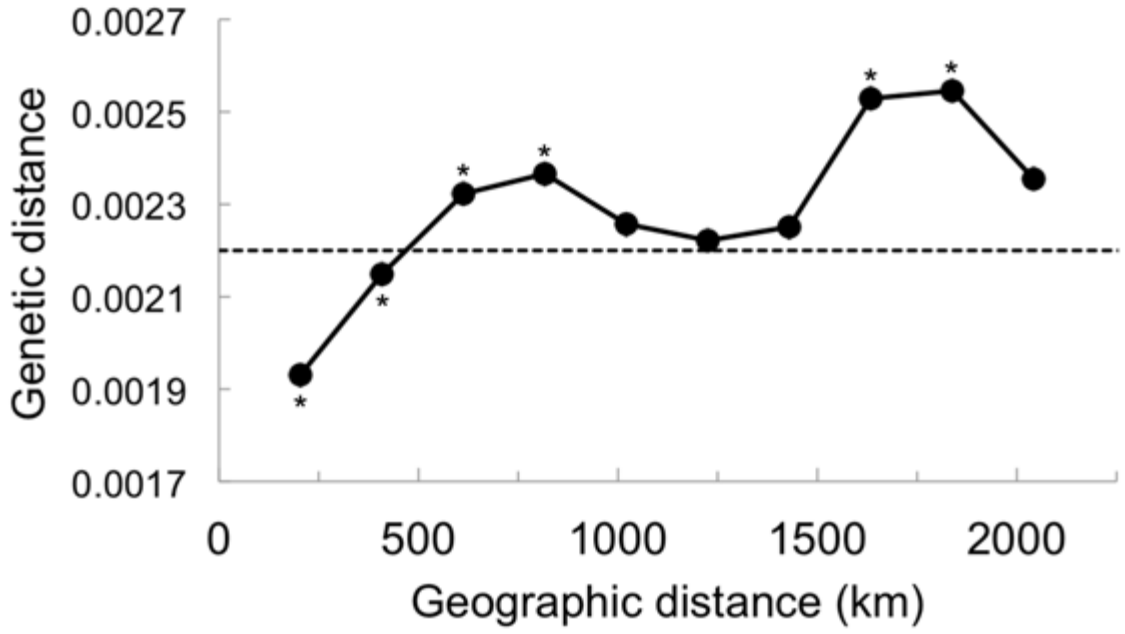
Genetic distance
0.0027
0.0025
0.0023
0.0021
0.0019
0.0017
0
500
1000
1500
2000
Geographic distance (km)

Fig. 6.

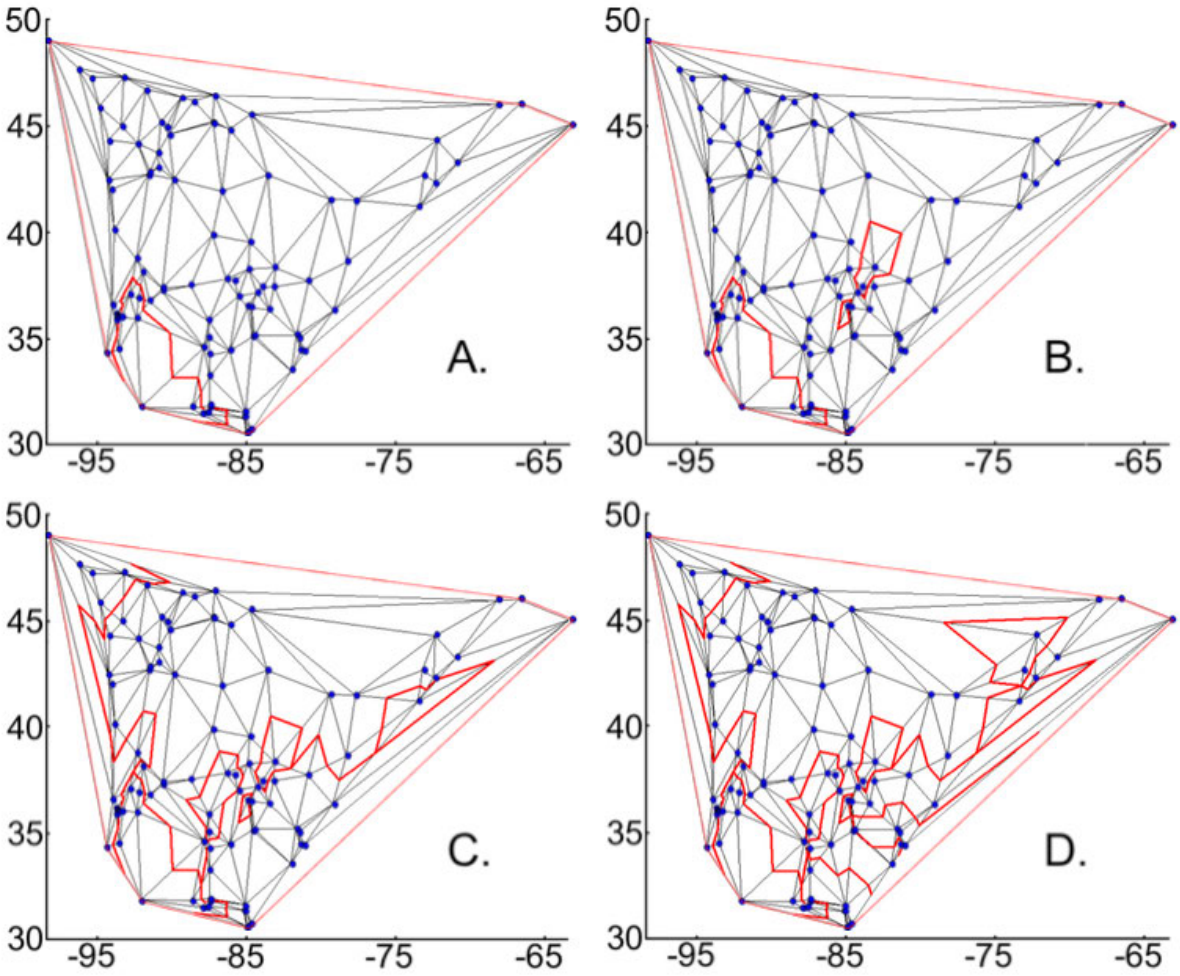

Fig. 7.

Figure Captions

Fig. 1. One hundred three populations of *Dirca* from which samples were collected for chloroplast sequencing. Samples also were collected from a population of *D. occidentalis* in the San Francisco Bay Area in California.

Fig. 2. Predicted climate suitability for *Dirca palustris* based on niche modelling in MaxEnt. A. Sixty-seven populations used for maximum-entropy modelling after conducting spatial filtering and balancing to reduce spatial bias. B. Projected distribution of suitable climate for the present. C. Projected distribution of suitable climate during the last glacial maximum based on CCSM climate reconstruction. D. Projected distribution of suitable climate during the last glacial maximum based on MIROC climate reconstruction. Coastlines in panels C and D extend beyond contemporary coastlines because sea levels were lower during the LGM.

Fig. 3. A. Chloroplast haplotype network for *Dirca.* Coloured circles are shared haplotypes (S-#) present in at least two populations each, with size of each circle corresponding to number of populations in which that haplotype was found. Small white circles are population-specific haplotypes (P-#s from Table 1). Number of line segments connecting each circle is the number of nucleotides that differ between haplotypes. Numbers and colours correspond to those on the map in Fig. 4. *Dirca occidentalis* differed substantially from the remaining species of *Dirca*, with 232 nucleotides differing between it and the next node. B. Bayesian phylogenetic tree of *Dirca* based on sequence data from five noncoding chloroplast regions. Coloured triangles are moderately to well-supported clades (posterior probability ≥ 0.70) coloured according to the

most common haplotype contained within them; coloured circles embedded to right of some clades are other shared haplotypes also found within them. Population-specific haplotypes (P-#s) contained within each clade are listed to the right of the clade. Asterisks indicate clades with posterior probability >0.96; other clades with posterior probability > 0.70 are denoted by values above branches. Numbers below branches indicate estimated ages of each node (M = million years; K = thousand years). Lengths of the two branches from *D. occidentalis* to the rest of the tree have been reduced for depiction.

Fig. 4. Map of haplotype distribution of *Dirca* in eastern North America. Each colour is a distinct haplotype shared among populations (S-#s from Fig. 3A), and each numbered white circle is a population-specific haplotype (numbered white circles from Fig. 3A; P-#s from Fig. 3B). Circles with more than one identity are populations for which polymorphism was detected among the three samples per population. Sequencing of seven additional plants in each of 11 populations revealed a third haplotype in two of them; three haplotypes are shown on the map for these populations.

Fig. 5. A. Locations of the southernmost occurrence of each shared haplotype or the singular occurrence of each population-specific haplotype from Fig. 3. B. Haplotypic richness of populations grouped by latitude (14 populations per group) from south to north. Richness = (no. haplotypes in group) / (35 haplotypes total) x (100). The polynomial curve is described by the formula $y = -0.07x^2 + 5.56x - 98.87$ ($R^2 = 0.35$).

Fig. 6. Results of spatial autocorrelation using ten distance classes ranging from a shortest class of 0-200 km to a longest class of 1840-2040 km. The dashed line is the average pairwise genetic distance among all individuals. Asterisks indicate distances that are greater or less than the average ($P<0.05$).

Fig. 7. Results of spatial genetic analysis by Monmonier's algorithm with number of barriers set to one, three, six, and nine (panels A, B, C, and D, respectively) for visualization of putative landscape barriers. Blue circles are sampled populations. Red lines are barriers identified by the algorithm. X-axis is longitude and y-axis is latitude of each sampled point.

CHAPTER 3. ISOLATION AND USE OF MICROSATELLITE MARKERS FOR A WOODY PLANT BY ILLUMINA PAIRED-END SEQUENCING, FLUORESCENT TAGGING WITH UNIVERSAL PRIMERS, AND MULTIPLEXED GENOTYPING

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Key words: co-dominant, *Dircа palustris* L., eastern leatherwood, enrichment, genetic marker, HiSeq, polymorphism, population genetics, simple sequence repeats, Thymelaeaceae.

Abstract

Researchers with limited budgets and equipment may be unable to isolate and genotype microsatellite loci from plants. Because next-generation sequencing is an economical approach to *de novo* identification of microsatellite loci, we evaluated the suitability of the Illumina Hiseq 2500 platform for isolation of microsatellites from genomic DNA of a shrub, *Dirca palustris* L. (Thymelaeaceae) without prior enrichment. We also evaluated the use of fluorescently tagged universal primers and multiplexed genotyping to further decrease costs. Sequence data from 1/5 of a single lane comprised 26.89 million fragments, 19,200 of which contained di-, tri-, or tetra-nucleotide simple sequence repeats (SSRs). The most common motifs were AT, ATT, and ATAC for di-, tri-, and tetra-nucleotide SSRs, respectively. Among all SSR fragments, 5,280 contained flanking sequences suitable for stringent primer design. Screening for copy number yielded 496 loci, most of which (89%) contained di-nucleotide SSRs. We screened 38 tri-nucleotide loci for amplification and polymorphism, which yielded 12 polymorphic loci among

plants from one population of *D. palustris*. Many loci transferred to the three other species of *Dirca* L. Several universal primers not originally designed for use with plants performed well with few nonspecific amplification products. Multiplexing amplification products into groups of four loci for genotyping generated a 75% savings in genotyping costs compared with genotyping amplification products individually. These approaches facilitate rapid and economical microsatellite identification and genotyping from plants with no prior genomic information available.

Introduction

Microsatellite (simple sequence repeats; SSR) markers are co-dominant genetic markers valued for their high level of polymorphism and ease of use. Genetic diversity measured by these markers is in the form of variation in the number of tandem repeats of a simple motif (one to several nucleotides), with variation introduced by DNA replication errors (Tautz and Renz 1984; Selkoe and Toonen 2006). Microsatellites are suitable for use by researchers with basic molecular biology tools for the study of genetic diversity across a range of scales from paternity analyses to population genetics and phylogeography. However, the time and expense of generating a set of microsatellites for a taxon without prior genomic information likely is prohibitive for some researchers (Selkoe and Toonen 2006). The process traditionally requires multiple steps, including enrichment of template DNA for SSRs, cloning, isolation of positive clones, Sanger sequencing, and primer design from flanking regions of suitable loci (Guichoux et al. 2011; Castoe et al. 2012).

Advances in next-generation sequencing (NGS) technologies have decreased both the cost and the time to generate a complete set of markers for non-model organisms (Faircloth

2008; Castoe et al. 2010, 2012; Guichoux et al. 2011). The Roche 454 (454 Life Sciences, Branford, CT, USA) and the Illumina HiSeq (Illumina, Inc., San Diego, CA, USA) are two NGS platforms useful for microsatellite isolation. NGS produces data from many individual DNA fragments sampled from the genome, some of which contain SSRs and enough flanking sequence for primer design. Roche 454 sequencing generally is preceded by an enrichment step to increase the abundance of microsatellite repeats in the DNA sample, as the sequencing of random genomic fragments on this platform is unlikely to yield enough potential loci (Lepais and Bacles 2011; Pandey and Sharma 2012). In contrast, the Illumina Hiseq platform produces several orders of magnitude more, but substantially shorter, individual reads. Despite its shorter read lengths, the Illumina platform is suitable for identifying thousands of potentially amplifiable SSR loci without prior enrichment (Castoe et al. 2012). Preparation of genomic samples for NGS is straightforward; template DNA is broken into strands of several hundred bases each, and a specific index adaptor is ligated to fragments before sequencing.

In addition to using next-generation sequencing for economical identification of potential microsatellite loci, researchers can reduce costs during primer screening and fluorescent genotyping. For instance, an alternative to the expensive practice of labeling a primer from each locus for fluorescent genotyping is to add a 5' tail to the forward primer for each locus and include a fluorescently tagged universal primer in the amplification reaction identical to the tail on the forward primer. During PCR, the universal primer takes over for the tailed forward primer when the latter is exhausted during PCR cycles (Fig. 1; Schuelke 2000; Missiaggia and Grattapaglia 2006; Blacket et al. 2012). In this way, genotyping of numerous loci can be accomplished with the synthesis of only one fluorescently tagged primer.

Another way to reduce costs is to multiplex numerous loci, either for genotyping after PCR has been completed or for both PCR and genotyping (Blacket et al. 2012; Culley et al. 2013). Multiplexing individually amplified PCR products for genotyping is advantageous for small- to mid-scale projects because amplification of single loci does not require additional troubleshooting associated with multiplexed PCR. In contrast, the latter approach is well suited for large-scale genotyping projects, as amplifying numerous loci in a single PCR reaction and subsequently genotyping them requires less time and materials when many loci or individuals must be genotyped (Lepais and Bacles 2011; Blacket et al. 2012; Culley et al. 2013). Regardless of which multiplexing strategy is selected, it is essential to plan the multiplex to include amplification products that either do not overlap in size or are tagged with distinct fluorophores.

The genus *Dirca* (leatherwood) comprises four species of understory shrubs indigenous to North America. Almost no genomic information is available for members of this genus, making *de novo* isolation of microsatellite markers challenging. The most broadly distributed species, *Dirca palustris* L., has a patchy distribution extending from Nova Scotia west to North Dakota, and south to Louisiana and Florida (Peterson et al. 2011). *Dirca occidentalis* Gray is endemic to six counties around the San Francisco Bay in California; *Dirca mexicana* Nesom & Mayfield is known from a single location in the Sierra Madre Oriental of northeastern Mexico; and *Dirca decipiens* Floden & Mayfield was described recently from one population in Kansas and two in Arkansas (Nesom and Mayfield 1995; Floden et al. 2009). Although broad in distribution, *D. palustris* has no obvious means for long-distance seed dispersal (Ward and Horn 1998; Peterson and Graves 2011) and is absent from many apparently suitable habitats throughout its range. The restricted distribution of three species of *Dirca* makes the broad, sporadic distribution of *D. palustris* especially intriguing. Identification of microsatellite (simple

sequence repeats; SSR) markers from *Dirca* could be used to study population genetics within the three narrowly endemic species of *Dirca* and might clarify the extent of seed dispersal and gene flow within the more widespread *D. palustris*.

We applied the Seq-to-SSR approach described for birds and reptiles by Castoe et al. (2012) to identify microsatellite loci from *D. palustris* on the Illumina HiSeq 2500. We evaluated the utility of several universal primers (Blacket et al. 2012) for fluorescent tagging of amplification products. Finally, we evaluated the potential to multiplex PCR products to facilitate fluorescent genotyping of multiple loci in one sequencing capillary.

Materials and Methods

DNA was extracted from an immature leaf of a single individual of *D. palustris* indigenous to Liberty Co., FL, by using the Wizard SV Genomic DNA Purification System (Promega Corporation, Madison, WI, USA). A DNA library with fragments of ~200-500 bases was prepared by the DNA Facility at Iowa State University, Ames, IA, and sequenced in the Rapid Mode of the Illumina HiSeq 2500 platform. We used the Perl script PAL_FINDER_v0.02.03 (Castoe et al. 2012) to search 26.89 million paired-end reads (two .fastq files) for potential loci with 2-, 3-, or 4-mer repeat motifs and a minimum of 12, 10, or 8 repeat units, respectively. Poor-quality priming sites were masked from flanking sequences by using the RepBase v14.01 database (Jurka et al. 2005; Castoe et al. 2012), and primer pairs were designed with a local installation of Primer3 2.0.0 (Rozen and Skaletsky 2000). We used the primer selection criteria of Castoe et al. (2012) and addressed concerns of copy number by screening potentially amplifiable loci (PALs) for the occurrence of their priming sites throughout the reads. We considered for further investigation only the loci for which at least one priming

site was unique across all reads and the other priming site occurred no more than twice.

To evaluate the usefulness of loci identified by Illumina sequencing, DNA was extracted (Edwards et al. 1991) from 14 plants in a single population of *D. palustris* from Grainger County, TN (36.402149, -83.45614). Extracted DNA was purified of contaminants by using the high-salt ethanol precipitation method of Fang et al. (1992) and diluted and stored in TE buffer at -20 C. First, 38 pairs of primers for tri-nucleotide SSRs selected after screening PALs were synthesized and evaluated for amplification and polymorphism within three individuals of *D. palustris*. The forward primer of each locus was synthesized with one of three tails on the 5' terminal identical in sequence to one of three universal primers labeled with a fluorophore (Table 1). Reactions of 10 µL each contained 5 µL of 2X GoTaq® Colorless Master Mix (Promega Corporation, Madison, WI, USA), 0.1 µM forward tailed primer, 0.2 µM reverse primer, 0.2 µM universal primer, and 25 ng of template DNA. Reaction conditions were 95°C for 10 minutes; followed by 12 cycles of 95°C for 30 seconds, 63°C with a ramp of -0.3°C per cycle for 90 seconds, and 72°C for 60 seconds; and 34 cycles of 95°C for 30 seconds, 59°C for 90 seconds, and 72°C for 60 seconds; and a final extension step of 72°C for 5 minutes. Amplification products were genotyped on an Applied Biosystems 3730 DNA Analyzer (Life Technologies Corporation, Carlsbad, CA). Amplifiable loci subsequently were screened for polymorphism across all 14 plants of *D. palustris* by conducting PCR individually for each locus and multiplexing into groups of four loci for genotyping. Minor troubleshooting allowed us to optimize concentrations of each amplification product for the multiplex. Polymorphic loci were screened across the three other species of *Dirca* to determine cross-amplification potential.

General characteristics of loci, including number of alleles, range of allele sizes, observed heterozygosity, expected heterozygosity under Hardy-Weinberg equilibrium, and fixation

statistics, were calculated by using GenAlEx 6.1 (Peakall and Smouse 2006). We used Genepop 4.2 (Raymond and Rousset 1995; Rousset 2008) to test for Hardy-Weinberg equilibrium and linkage disequilibrium.

Results

Analysis of sequence data from 26.89 million fragments yielded 19,200 fragments (0.07%) containing candidate loci with di-, tri-, or tetra-nucleotide SSRs (Table 2). Among di-nucleotide SSRs, 61% had a motif of AT, 30% were TC, 9% were AC, and 0% were CG. Among tri-nucleotide SSRs, 45% had a motif of ATT, 32% were TTC, 14% were ATC, and each remaining motif accounted for less than 5% of tri-nucleotide SSRs. Among the comparatively few tetra-nucleotide repeats identified, 38% had a motif of ATAC, 19% were AATG, 16% were ATCT, and each remaining motif accounted for less than 10% of tetra-nucleotide SSRs (Table 2). Of the 19,200 candidate loci identified, 5280 (27.5%) contained enough high-quality flanking region for primer design (Table 2). Screening these PALs for copy number yielded 496 stringently filtered PALs suitable for further testing. Of these, 440 contained simple di-nucleotide motifs, 43 contained simple tri-nucleotide motifs, four contained simple tetra-nucleotide motifs, and nine contained broken di-nucleotide motifs.

Of the 38 tri-nucleotide SSR loci screened, 12 (31.6%) were consistently amplifiable (Table 3). All were polymorphic within the population of *D. palustris* (Table 4). Number of alleles per locus varied from three to 12, observed heterozygosity ranged from 0.43 to 0.92, and expected heterozygosity ranged from 0.36 to 0.87. The fixation index for each locus ranged from -0.09 to 0.38 (Table 4). A test for deviation from Hardy-Weinberg equilibrium found evidence for deviation in only two loci (C and G; Table 4), and tests for linkage disequilibrium

found no evidence that loci were linked (*P* ranged from 0.07 to 1.00 across all pairwise comparisons). Four loci amplified in all four species of *Dirca,* and all loci amplified in at least one species other than *D. palustris* (Table 5).

Discussion

Next-generation sequencing of *D. palustris* demonstrated the effectiveness of paired-end sequencing without enrichment on the Illumina HiSeq 2500 in the identification of thousands of SSR regions with suitable priming sites from a non-model woody plant. Although this approach was demonstrated to be suitable for isolation of microsatellites from animal taxa, even those with a low frequency of genomic SSRs (Castoe et al. 2012), our results expand on their findings by demonstrating the utility of this sequencing platform to isolate microsatellite loci from plants. We isolated 12 polymorphic loci from *D. palustris*, many of which were transferable to the other species of *Dirca.* The speed and low cost of NGS, coupled with the use of fluorescently tagged universal primers and multiplexing of numerous loci for genotyping, put the widespread use of microsatellite genetic markers within reach for many plant scientists.

This approach to microsatellite isolation required little investment of time and money to generate numerous candidate loci. The cost of DNA extraction, library preparation, and sequencing on an entire lane of the HiSeq 2500 was less than $2,200. Computer processing time of .fastq files with the Perl script PAL_FINDER_v0.02.03 in conjunction with Primer3 2.0.0 was ~36 hours on a 2.53-GHz Intel Core 2 Duo MacBook Pro running Mac OSX. Our analysis of 26.89 million paired-end reads (less than 1/5 of a lane) demonstrates that a fraction of a lane is sufficient to generate hundreds of loci with high-quality priming sites from woody angiosperms with SSR abundance similar to that of *Dirca*. Our results suggest that a smaller fraction of a

sequencing lane could be used for identification of hundreds of candidate loci, but, for us, the number of tri- and tetra-nucleotide SSRs was limiting. In the event that less than 1/5 of a sequencing lane is used, lowering the requirements for SSR repeat number in the *PAL_FINDER* script by modestly relaxing copy-number requirements when filtering candidate loci could increase the number of tri- and tetra-nucleotide SSR loci. Finally, although we did not evaluate its use, we suspect the Illumina MiSeq, which produces many more reads than does the Roche 454 and longer read lengths does than the HiSeq, would be suitable to identify microsatellite loci in one to several taxa indexed in the same lane, without enrichment.

The loci we isolated were consistently amplifiable, polymorphic, and showed no evidence of linkage disequilibrium, demonstrating that data from the Seq-to-SSR approach of Castoe et al. (2012) leads to rapid identification of high-quality microsatellite loci in plants. Moreover, the high variation (up to 12 alleles) observed at multiple loci suggests that these markers may have power suitable for resolving questions that range in scale from population genetics to paternity testing among closely related individuals (Selkoe and Toonen 2006). Cross-amplification of many loci to other congeners also indicated these markers could be useful in studies at the infrageneric level (Table 5).

The use of universal primers and the multiplexing of amplification products for genotyping also contributed to the economy of our protocols. The use of tailed forward primers and universal primers decreased the cost of fluorescently tagging PCR products by hundreds of dollars. Moreover, the three universal primers we synthesized are suitable for additional work with unrelated taxa, generating future cost savings. Multiplexing amplification products into sets of four decreased the costs of genotyping by 75%. Together, Illumina paired-end sequencing, universal primers, and multiplexing enable nearly any researcher with access to basic molecular

biology equipment and a DNA core facility to develop microsatellite markers for non-model organisms and genotype 500 individuals across 8-10 loci for less than $10,000 USD.

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Table 1. Universal primers and fluorescent tags used for genotyping of 12 microsatellite loci from *Dirca palustris*. Tail sequences of universal primers are identical to tails on the 5' ends of locus-specific forward primers.

Universal primer	Tail sequence (5'-3')	Fluorescent dye
Tail A	GCCTCCCTCGCGCCA	6-FAM
Tail B	GCCTTGCCAGCCCGC	HEX
Tail C	CAGGACCAGGCTACCGTG	NED

Table 2. Characteristics of SSRs obtained from *Dirca palustris* by using paired-end sequencing on the Illumina HiSeq 2500 platform, including identity of repeat motifs, number of potential loci with each motif, and number of loci with suitable priming sites. Only motifs present at one or more loci are shown (e.g., no loci with the di-nucleotide GC motif were identified). Repeat motifs were condensed automatically by PAL_FINDER_v0.02.03; for instance, the motif AC includes loci with the equivalent CA motif, as well as the genetic complements TG and GT.

Repeat motif	Loci	Loci with priming sites
AC	1500	357
AT	10336	2279
TC	5103	2081
AAC	82	28
ACC	9	4
AGT	50	4
ATC	268	70
ATT	869	117
TCC	21	12
TCG	11	0
TGC	1	0
TTC	607	241
AAAC	10	2
AAAG	30	20
AAAT	14	2
AACT	4	1

Table 2 continued

AATG	66	21
AATT	6	0
AGGG	2	0
AGGT	1	0
AGTG	3	1
ATAC	132	31
ATCT	54	5
ATGG	19	4
TCTG	2	0

Table 3. Primers for 12 polymorphic loci identified for *Dirca palustris*. Amplification products from each locus were multiplexed into one of three sets of loci for genotyping. Tails (A, B, or C) on forward primers correspond to those from Table 1. Multiplex concentrations are by volume.

Locus	SSR motif	Tailed forward primer	Reverse primer	Multiplex concentration
Multiplex 1				
A	ATT(15)	B__TATGCAAGGGAAAACACAACC	AAAACCCTTAAGGTTGGGACC	0.50
B	TTC(12)	A__TTTGGAAAATGGAAGAATAACAGC	GACAATGTTGGTCCCCTGC	0.40
C	TTC(10)	C__AAAGGAAATTACACCCCAATCC	GGCGTTTTCAATGTTTTCTGC	0.05
D	ATT(10)	C__TGGAACTGTTACAACACATGTACG	ACGGGCTAGACGGGTTAGG	0.05
Multiplex 2				
E	ATC(17)	C__GATTCTGTTTGCCCGTTCG	GCGTTTGGAGGTTAGAAGACC	0.05
F	ATT(15)	B__AAAATCATGCAAATCACTAACATGC	TGCTGAGTTTAGCCGAATGC	0.45
G	TTC(12)	A__GGCATAATTCACGGTGAGACC	GAGATCAACTTTGAGTCTTGAAACC	0.45
H	ATT(11)	C__AACGAACGAAACCTCGATCC	GAAACATAGTGACAAGTTTGCCC	0.05

Table 3 continued

Multiplex 3

I	ATT(20)	B__CGAAAGAATTCACTTCCCCG	GTGGCGTCTGTCCTAATCTACC	0.40
J	ATT(12)	A__GAAGAAGGGCTGCTTTGACC	AATCGAATGAACTTCCATGACC	0.45
K	ATT(11)	C__AATTGCCATGCATGTTGC	AGAGTATGCGTGAAGCGTCC	0.10
L	ATT(10)	C__GATCCACAGAGGATTCCCG	CATCCATCAGTCCTATTAAGTCGC	0.05

Table 4. Characteristics of 12 loci amplified by using genomic DNA from 14 individuals in a population of *D. palustris* in Grainger Co., TN. N = number of individuals for which the locus amplified; alleles = number of alleles detected; bp = range of allele sizes in basepairs; $H_{(obs)}$ = observed heterozygosity; $H_{(exp)}$ = expected heterozygosity under Hardy-Weinberg equilibrium; F_{IS} = fixation index; HWE = *P*-values for tests of deviation from Hardy-Weinberg equilibrium.

Locus	N	Alleles	bp	$H_{(obs)}$	$H_{(exp)}$	F_{IS}	HWE
A	13	12	292-337	0.85	0.87	0.03	0.253
B	14	4	227-251	0.71	0.72	0.01	0.183
C	14	10	178-205	0.57	0.84	0.32	0.002
D	11	6	485-512	0.58	0.77	0.24	0.110
E	14	3	421-430	0.43	0.36	-0.20	1.000
F	14	7	207-237	0.71	0.81	0.12	0.145
G	14	10	221-251	0.50	0.80	0.38	0.010
H	14	6	227-251	0.71	0.73	0.03	0.947
I	14	10	185-218	0.92	0.84	-0.09	0.591
J	13	7	181-202	0.75	0.82	0.08	0.487
K	12	7	470-497	0.64	0.75	0.15	0.087
L	14	9	208-253	0.71	0.83	0.14	0.236

Table 5. Cross-amplification of loci to the three other species of *Dirca*. *Dirca mexicana* was collected from Tamaulipas, Mexico (23.98583, -99.47694); *D. decipiens* was collected from Johnston Co., KS (38.795838, -94.691634); and *D. occidentalis* was collected from San Mateo Co., CA (37.4075, -122.23605). Y indicates amplification, and -- indicates no amplification.

Locus	*D. mexicana*	*D. decipiens*	*D. occidentalis*
A	Y	Y	--
B	Y	Y	--
C	Y	Y	Y
D	Y	--	Y
E	Y	Y	Y
F	Y	--	--
G	Y	Y	--
H	--	--	Y
I	Y	Y	Y
J	Y	--	Y
K	Y	--	--
L	Y	Y	Y

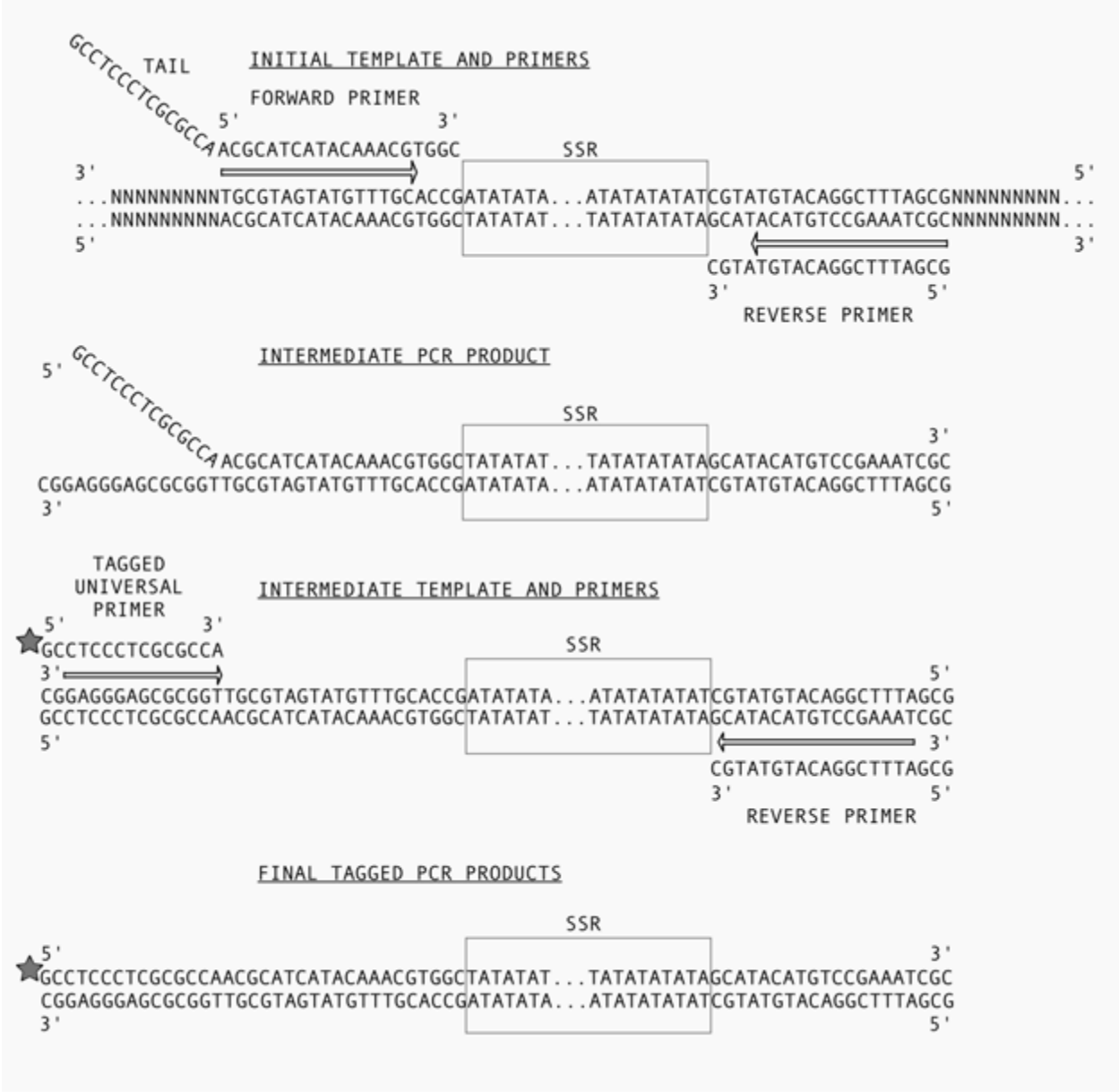
TAIL
GCCTCCCTCGCGCCA
INITIAL TEMPLATE AND PRIMERS
FORWARD PRIMER
5'
3'
ACGCATCATACAAACGTGGC
SSR
3'
5'
...NNNNNNNNNTGCGTAGTATGTTTGCACCGATATATA...ATATATATATCGTATGTACAGGCTTTAGCGNNNNNNNNN...
...NNNNNNNNNACGCATCATACAAACGTGGCTATATAT...TATATATATAGCATACATGTCCGAAATCGCNNNNNNNNN...
5'
3'
CGTATGTACAGGCTTTAGCG
3'
5'
REVERSE PRIMER
INTERMEDIATE PCR PRODUCT
5'
GCCTCCCTCGCGCCA
SSR
3'
ACGCATCATACAAACGTGGCTATATAT...TATATATATAGCATACATGTCCGAAATCGC
CGGAGGGAGCGCGGTTGCGTAGTATGTTTGCACCGATATATA...ATATATATATCGTATGTACAGGCTTTAGCG
3'
5'
TAGGED
UNIVERSAL
PRIMER
INTERMEDIATE TEMPLATE AND PRIMERS
5'
3'
GCCTCCCTCGCGCCA
SSR
3'
5'
CGGAGGGAGCGCGGTTGCGTAGTATGTTTGCACCGATATATA...ATATATATATCGTATGTACAGGCTTTAGCG
GCCTCCCTCGCGCCAACGCATCATACAAACGTGGCTATATAT...TATATATATAGCATACATGTCCGAAATCGC
5'
3'
CGTATGTACAGGCTTTAGCG
3'
5'
REVERSE PRIMER
FINAL TAGGED PCR PRODUCTS
SSR
5'
3'
GCCTCCCTCGCGCCAACGCATCATACAAACGTGGCTATATAT...TATATATATAGCATACATGTCCGAAATCGC
CGGAGGGAGCGCGGTTGCGTAGTATGTTTGCACCGATATATA...ATATATATATCGTATGTACAGGCTTTAGCG
3'
5'

Fig. 1

Figure Captions

Fig. 1. Example of templates, primers, and PCR products in microsatellite genotyping by fluorescence with universal primers. A. Initial DNA template with a di-nucleotide SSR of the AT motif. Locus-specific primers bind to priming sites and amplification of target sequence is achieved by PCR. Arrows indicate direction of replication, which is initiated at the 3' end of each primer. The forward primer of each locus has a 5' tail to facilitate fluorescent tagging with a universal primer during subsequent PCR cycles. B. Intermediate PCR products are amplified by locus-specific primers, with tail sequence (or complement) incorporated into each strand of the products. The tailed locus-specific primer is exhausted during successive rounds of PCR. C. Products amplified in early rounds of PCR become templates for subsequent amplifications utilizing a universal primer. The universal primer tagged on the 5' end with a fluorophore (shown here as a star) becomes incorporated into PCR products during subsequent amplification cycles. D. Final PCR products are successfully tagged with a fluorescent dye, without synthesizing a unique fluorescently tagged forward primer for that locus. Length (bp) of each amplification product is determined by the number of tandem repeats, with different alleles having different lengths.

CHAPTER 4. POPULATION GENETICS OF *DIRCA PALUSTRIS* L. SUPPORT THE EXISTENCE OF MID-LATITUDINAL REFUGIA DURING THE LAST GLACIAL MAXIMUM IN EASTERN NORTH AMERICA

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Keywords: cryptic refugia, eastern leatherwood, gene flow, genetic diversity, microsatellites, migration, phylogeography, Reid's paradox, seed dispersal, simple sequence repeats, SSR.

Abstract

Dirca palustris is a shrub indigenous to mesic forest understories of eastern North America, where it occurs in scattered, highly localized populations from Florida to North Dakota and Nova Scotia. Despite its broad distribution, no agents for long-distance dispersal of its seeds have been identified. To help resolve this paradox, we used microsatellite genetic markers to evaluate the hypothesis that *D. palustris* persisted not only in southern refugia, but also in mid-latitudinal refugia closer to the continental ice sheet and more central to its current range, during the Last Glacial Maximum (LGM). STRUCTURE analysis of microsatellite alleles showed two distinct genetic clusters of populations, one at lower latitudes, and the other at middle to higher latitudes. Mantel tests and tests of spatial autocorrelation demonstrated strong dispersal limitation at local scales, with occasional long-distance dispersal. Populations tended to have high inbreeding coefficients (F_{IS}), indicative of limited local gene flow. Range expansion into higher latitudes following the LGM was not associated with a loss of genetic diversity, suggesting a repeated dispersal of alleles from middle latitudes to the expanding edge of the

range during colonization. We speculate that the now-extinct passenger pigeon (*Ectopistes migratorius*), which likely was responsible for northward range expansion of many forest taxa following the LGM, also occasionally transported seeds of *D. palustris* during migration to breeding grounds in the Great Lakes Region. The results of this study lend additional support to previous conclusions based on chloroplast phylogeography.

Introduction

Temperate plants indigenous to eastern North America migrated and persisted through cyclical advances and retreats of glaciers during Pleistocene climate oscillations. The most recent glacial maximum was ~20,000 years ago, when the Laurentide ice sheet occupied much of the Upper Midwest, the Great Lakes Region, and the Northeast (Comes and Kadereit 1998; Hewitt 2004). The contemporary distribution of plant taxa in eastern North America depends both upon the locations of suitable habitats where they persisted during the LGM, and the frequency and distance of dispersal during the current interglacial. Phylogeographic and population-genetic studies involve various techniques to clarify where taxa persisted during the LGM and the extent of gene flow and dispersal among extant populations.

Traditionally, scientists have believed that temperate forest elements were shifted far to the south (below ~34°N) during the LGM and that boreal forest elements occupied habitats closer to the Laurentide ice sheet (Delcourt and Delcourt 1998; Soltis et al. 2006). However, boundaries delineating vegetation assemblages during the LGM are disputed, and accumulating genetic evidence suggests a more heterogeneous assortment of boreal and temperate taxa at middle latitudes (Jackson et al. 2000; Rowe et al. 2004; McLachlan et al. 2005; Soltis et al. 2006; Loehle 2007; Gonzales et al. 2008). The persistence of temperate species in cryptic northern

refugia, where isolated or scattered populations were too scarce to imprint the pollen record (Jackson et al. 2000), may be critical to understanding the contemporary distribution of temperate species in eastern North America. The observation that the ranges of many species extend further north than predicted by inferred capacities for seed dispersal from hypothesized southern refugia (Clark et al. 1998) could be explained in part by the persistence of temperate taxa further north than traditionally hypothesized for the LGM.

Two complementary approaches to studying the phylogeography of plants include sequencing of plastid DNA and genotyping with nuclear genetic markers. Plastid sequencing is suitable for inferences about deep Pleistocene history and historical patterns of persistence and seed dispersal because mutation rates are low and inheritance typically is maternal. In contrast, nuclear genetic markers (e.g., microsatellites) can be used to make inferences about population structure, seed dispersal, and gene flow by pollen or seeds in the more recent past. Here, we build on a previous study in which we investigated the chloroplast phylogeography of *Dirca palustris* L. (Peterson 2013) by extending our investigations to the use of microsatellite genetic markers. Microsatellites (simple sequence repeats; SSRs) are co-dominant genetic markers valued for their high degree of polymorphism and straightforward genotyping. Genetic diversity measured by these markers is assessed by variation in the number of tandem repeats of a simple motif (1 to several nucleotides), with variation introduced by errors in DNA replication (Tautz and Renz 1984; Selkoe and Toonen 2006). The informational content of microsatellites often is sufficient to enable inferences about population-genetic processes based on several loci, especially when loci are hypervariable (Kalinowski 2002). In this study, we amplified eight highly polymorphic microsatellite loci that we isolated from *D. palustris* (Peterson 2013).

Dirca L. comprises four named species of deciduous shrubs indigenous to North America. *Dirca palustris* inhabits rich, mesic understories throughout eastern North America, whereas *Dirca occidentalis* Gray is known from six counties around the San Francisco Bay in California, *Dirca mexicana* Nesom & Mayfield was described from a single population in northeastern Mexico (Nesom and Mayfield 1995), and *Dirca decipiens* Floden & Mayfield was discovered and named from populations on the western edge of the range of *D. palustris* (Floden et al. 2009). Although broadly distributed, *D. palustris* occurs sporadically across the landscape, often is absent from apparently suitable habitats, and lacks obvious vectors for long-distance dispersal (Ward and Horn 1998; Peterson and Graves 2011). In the southern portion of its distribution, populations usually are small and highly localized in moist, scattered niches (Nevling 1962; Ward and Horn 1998); in contrast, populations tend to be more common throughout hardwood forests of the Great Lakes Region of northern Wisconsin and the Upper Peninsula of Michigan, where the species sometimes is a dominant member of the shrub community (Schulz et al. 2004).

Our previous study on the chloroplast phylogeography of *D. palustris* supported the hypothesis that the species persisted in mid-latitudinal refugia during the LGM, which helped to resolve its paradoxical occurrence at high latitudes despite the absence of obvious vectors for long-distance dispersal of seeds (Peterson 2013). Southern populations were an unlikely source of propagules colonizing northern latitudes. We also found a relatively high diversity of chloroplast haplotypes in the Upper Great Lakes Region, which suggested that repeated instances of long-distance dispersal created a genetically heterogeneous patchwork of regional populations.

Our objectives were to use microsatellite markers to 1) further evaluate the plausibility of mid-latitudinal refugia suggested by chloroplast phylogeography, 2) test the hypothesis that dispersal and gene flow among populations of *D. palustris* prehistorically were limited, and 3) evaluate the hypothesis that northward migration was associated with a decrease in genetic diversity indicative of genetic bottlenecks and founder effects.

Materials and Methods

We collected one leaf from each of 579 plants of *D. palustris* in 39 populations and dried them in silica-gel desiccant (Table 1). One voucher specimen from each population was pressed, dried, and deposited in ISC (Thiers 2013). Although we originally intended to genotype samples from the three other species of *Dirca,* the failure of many loci to cross-amplify (Peterson 2013) and the likelihood for homoplasy among remaining loci, which were hypervariable within *D. palustris*, led us to restrict our analysis to *D. palustris*. DNA was extracted (Edwards et al. 1991) and purified (Fang et al. 1992), and microsatellite loci A-H from Peterson (2013) were amplified and genotyped following the steps described therein. Genotyping was conducted with an Applied Biosystems 3730 DNA Analyzer (Life Technologies Corporation, Carlsbad, CA), and alleles were scored by fragment length with the Microsatellite Plugin 1.3.2 in Geneious® 6.1.6 (Biomatters Ltd., Auckland, New Zealand).

We used the web-based version of Genepop 4.2 (Raymond and Rousset 1995; Rousset 2008) to conduct various analyses on the microsatellite data. We tested for linkage disequilibrium (LD) in pairwise comparisons of loci, and one of two loci was discarded if the two were significantly correlated after Bonferroni correction for multiple comparisons. Hardy-Weinberg equilibrium (HWE) was tested across all populations and loci, and both the inbreeding

coefficient (F_{IS}) and tests for heterozygote deficiency were calculated for each population to identify local inbreeding. Fstat v. 2.9.3 (Goudet 2001) was used to calculate allelic richnesss (total alleles identified per population) and gene diversity (unbiased estimator of expected heterozygosity per locus per sample; Nei 1987). We also visualized general relationships among populations based on Cavalli-Sforza genetic distances (Cavalli-Sforza and Edwards 1967) by constructing an unrooted neighbor-joining tree in the NEIGHBOR program of PHYLIP (Felsenstein 2005) with 999 bootstrap permutations.

STRUCTURE 2.3.4 (Pritchard et al. 2000) was used to identify genetic discontinuities in the microsatellite data without *a priori* specification of population groupings. The software implements a Bayesian clustering algorithm to assign probabilities that individuals belong to each of *K* genetic groups specified by the user. For the analysis, we assumed correlated allele frequencies and admixed ancestry; the burn-in was set to 20,000 generations followed by 50,000 Markov Chain Monte Carlo iterations. We evaluated *K* from 2 to 10, with 10 replicate analyses each. The Δ*K* approach of Evanno et al. (2005) was used in STRUCTURE HARVESTER web v.0.6.93 (Earl and vonHoldt (2012) to identify the optimal number for *K*. We used CLUMPP v. 1.1.2 (Jakobsson and Rosenberg 2007) to convert results from 10 replicate runs of the optimal *K* into single probabilities of cluster assignment for each individual and population. A map was constructed depicting the probability of assignment for each population to one of *K* clusters.

Analysis of molecular variance (AMOVA) was conducted in GenAlEx 6.1 (Peakall and Smouse 2006) to assess the amount of total genetic variation partitioned among populations, and to evaluate several specific hypotheses. The first hypothesis we evaluated was *K* genetic clusters identified by STRUCTURE, and we also tested hypothesized barriers to east-west gene flow by the combined influence of the Appalachian Mountain Range and the Apalachicola River, and by

the Mississippi River. General patterns of range-wide genetic structure were evaluated by conducting the Mantel test (Mantel 1967) for isolation-by-distance and by testing for spatial autocorrelation in which genetic similarity of individuals within 10-km distance classes were compared against a null hypothesis of no genetic structure. Both tests were conducted across the entire sampling distribution of *D. palustris* and separately in each of *K* regions defined by STRUCTURE. Allelic richness, gene diversity, and F_{IS} were plotted against latitude to test whether northward range expansion was associated with a loss of genetic diversity.

Results

Genetic analyses

Although all eight loci yielded amplification products from most plants throughout the range of *D. palustris*, approximately 5% of reactions failed to amplify loci. Missing data could be attributed largely to several populations where individual loci failed to amplify, indicative of population-wide mutations in priming sites. Tests for linkage disequilibrium with Bonferroni correction showed that locus G was correlated positively with three other loci. This locus was removed from further consideration, after which no other loci showed evidence for disequilibrium. Tests for deviation from Hardy-Weinberg equilibrium showed that all loci demonstrated a heterozygote deficiency across the species distribution (all *P*-values < 0.001), indicative of range-wide population subdivision (i.e., Wahlund effect; Hartl and Clark 1997). Among the 39 populations we investigated, the number of individuals genotyped per population ranged from six to 16 (mean = 14.8), alleles per population ranged from seven (AL2) to 51 (MI1), and 33 of the 39 populations (85%) had significant, positive inbreeding coefficients (Table 1).

We found evidence for spatial genetic structure in *D. palustris*. Bayesian analysis of population assignment by STRUCTURE identified K=2 clusters, with populations at lower and higher latitudes generally assigned to different genetic clusters (Fig. 1). AMOVA revealed that 52% of total genetic variation was partitioned among populations, and an AMOVA analyzing the northern and southern genetic clusters identified by STRUCTURE showed that 7.2% of the total genetic variation was among regions (Table 2). Two additional AMOVAs showed that 7.2% of total genetic variation was partitioned between groups separated by the putative east-west barrier of the Appalachian Mountain range and the Apalachicola River, whereas only 1.7% of genetic variation was partitioned between regions separated by the Mississippi River (Table 2). Despite this evidence for spatial genetic structure, neighbor-joining analysis did not show genetic clusters by region, and bootstrap values were low (Fig. 2).

Additional evidence for genetic structure in *D. palustris* was identified by tests of spatial and genetic correlation. A Mantel test revealed moderate ($r = 0.23$) range-wide isolation-by-distance ($P < 0.001$). Additional Mantel tests showed that populations in the southern group identified by STRUCTURE had a greater correlation between genetic and geographic distances ($r = 0.35$; $P < 0.001$) than did populations in the northern group ($r = 0.23$; $P = 0.01$). Spatial autocorrelation across the entire distribution of *D. palustris* revealed limited gene flow, as plants within 10 km of one another tended to be most genetically similar (Fig. 3). Beyond these distances, correlation coefficients oscillated between positive and negative values across various distance classes (Fig. 3). Separate spatial autocorrelation analyses within the northern and southern regions showed similar patterns of strong genetic similarity at the lowest distance class (maximum r values ~0.40), with oscillation between positive and negative coefficients across greater distances (not shown).

We found no evidence for the hypothesis that northward range expansion resulted in the loss of genetic diversity (Fig. 4). Weak positive correlations were observed between latitude and both allelic richness ($r^2 = 0.10$) and gene diversity ($r^2 = 0.15$), but not the degree of inbreeding ($r^2 = 0.01$).

Discussion

Dirca palustris indigenous to northern latitudes likely originated from propagules derived from individuals that survived the LGM in mid-latitudinal refugia, and not those that survived near the Gulf Coast (Fig. 1; Table 2). Although long-distance dispersal likely occurs occasionally, dispersal limitation is a primary determinant of the structure of populations throughout the range of *D. palustris* (Fig. 3). Expansion into northern latitudes was not associated with a loss of genetic diversity (Fig. 4), a finding consistent with repeated dispersal of alleles from middle latitudes to the expanding edge of the range.

Our conclusion that microsatellite alleles indigenous to northern latitudes were transported from mid-latitudinal refugia is concordant with results from our previous study of chloroplast phylogeography of *D. palustris* (Peterson 2013). In that study, distribution of chloroplast haplotypes indicated that southern refugia were not likely the sources of propagules that colonized northern latitudes. In the present study, plants in the North were genetically similar to those at middle latitudes, and distinct from those in the South (Fig. 1; Table 2). In addition to supporting conclusions by Peterson (2013) about the late-Pleistocene distribution of *D. palustris*, these results are consistent with findings by others that demonstrate some temperate forest taxa of eastern North America occupied habitats within several hundred km of the Laurentide ice sheet during the LGM (McLachlan et al. 2005; Soltis et al. 2006; Gonzales et al.

2008). The existence of mid-latitudinal refugia of temperate forest taxa is supported further by observations that regions like the Ozark Ecoregion, the Cumberland Plateau, and the southern Appalachian Basin in eastern North America contain many endemic taxa, presumed to be relictual populations that survived *in-situ* during the LGM (The Nature Conservancy 2003; Tribsch and Schönswetter 2003).

Locally restricted dispersal of seeds and pollen of *D. palustris* was demonstrated by the positive correlation between geographic and genetic distances detected by Mantel tests, and by the higher genetic similarity of plants within 10 km revealed by spatial autocorrelation (Fig. 3). Moreover, high levels of inbreeding detected within many populations (Table 1) illustrated that the sporadic occurrence of small populations across the landscape likely was prehistorical, and not the product of more recent fragmentation of broader populations. A peculiar aspect of our findings was the oscillation of positive and negative correlation coefficients across many distance classes in the spatial autocorrelation analysis (Fig. 3). A pattern of positive autocorrelation at shorter distances with oscillations at greater distances is indicative of a patchy, rather than clinal, distribution of alleles across the landscape (Sokal and Jacquez 1991; Peakall et al. 2003). Such patchiness can be attributed to a general dispersal limitation coupled with rare instances of long-distance dispersal that create pockets of related genotypes distributed throughout the landscape. This so-called stratified dispersal is characterized by slow diffusion of genes or individuals across the landscape coupled with occasional instances of long-distance dispersal (Bialozyt et al. 2006).

The stratified dispersal suggested by spatial genetic analyses is difficult to explain if we consider only the contemporary paucity of vectors likely to disperse *D. palustris* on scales exceeding meters or tens of meters (Ward and Horn 1998; Peterson and Graves 2011; Floden

2011). The role of gravity in dispersal of seeds of *D. palustris* is obvious, as individuals tend to be clumped within the landscapes of local populations, and mature plants often are surrounded by numerous seedlings with no or few seedlings evident in gaps between maternal clusters (Peterson and Graves 2011). Highly localized dispersal explains the extensive inbreeding and differentiation among discrete populations (Table 1; Table 2). Highly localized dispersal does not, however, easily explain the migration of the species across the landscape, including its dispersal to northern latitudes once covered by the continental ice sheet.

In the absence of contemporary vectors for long-distance seed dispersal of *D. palustris*, two plausible explanations exist for historical occasional long-distance dispersal of the species. In previous work (Peterson 2013), we suggested that the now-extinct passenger pigeon (*Ectopistes migratorius* L.) may have transported seeds tens or hundreds of kilometers during northward migrations to breeding grounds in the Great Lakes Region. The ecology of the passenger pigeon, including its annual migration from southern hardwood forests to northern latitudes in extraordinary abundance, its rapid flight, its generalist diet and tendency to forage on the forest floor, its capacity for delayed digestion, and its tendency to regurgitate low-quality food when more nutritious sustenance was encountered (Schorger 1955; Webb 1986; Ellsworth and McComb 2003), renders plausible the hypothesis that passenger pigeons occasionally transported viable seeds of *D. palustris* across the landscape.

Hydrochory is a weak alternative hypothesis for long-distance transport of seeds across the landscape. Williams (2009) found that the fruits of *D. palustris* exhibited only a limited capacity to remain afloat and noted that the timing of most vernal flooding events is asynchronous with fruit maturation. Furthermore, as rivers in eastern North America generally flow from north to south (i.e., high to low elevation), hydrochory seems unlikely to account for

the northward range expansion of *D. palustris* since the LGM, even if it explains some instances of long-distance dispersal of the species. Although *D. palustris* tends to be restricted to riparian systems in the southern portion of its distribution (Ward and Horn 1998; Floden 2011), it is not restricted in this way in northern hardwood forests (Schulz et al. 2004). Therefore, the limited distribution of species in the South may be more due to the presence of cool, moist niches with relictual populations near riparian systems than a result of hydrochory. Finally, the weak, positive correlation between latitude and both allelic richness and gene diversity (Fig. 4) provides additional support for the hypothesis that an active vector, rather than rare transport by water from south to north, facilitated repeated sampling of genetic diversity from refugial locations and dispersed it to the Upper Great Lakes Region. The similar degree of inbreeding in northern and southern populations also demonstrates that genetic bottlenecks and founder effects apparently did not diminish genetic diversity within populations established after the retreat of continental glaciers (Fig. 4).

The results of this study support conclusions similar to those based on the chloroplast phylogeography of *D. palustris*. When we consider the evidence from this and our earlier study, it is difficult to dismiss the hypotheses that an active vector (e.g., the passenger pigeon) dispersed seeds to northern latitudes following the LGM, and that northward dispersal during the current interglacial was from mid-latitudinal refugia.

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Table 1. Characteristics of populations sampled for microsatellite analysis. Measures include latitude and longitude, number of individuals sampled, number of alleles, inbreeding coefficient (F_{IS}), and *P*-value for a strict test of heterozygote deficit (i.e., inbreeding). Populations are sorted from south to north, and each population label includes the two-letter postal code of the state in which it occurs. Number of alleles sampled per population was summed across seven loci. Population AL2 had no inbreeding coefficient because all plants were monomorphic at each locus.

Population	Latitude	Longitude	Individuals	Alleles	F_{IS}	*P*-value
FL1	30.590637	-84.935303	15	18	0.39	0.006
FL2	30.626095	-84.902344	16	22	0.27	<0.001
GA1	31.353333	-85.065000	16	46	0.01	0.263
GA1	31.616136	-85.049303	14	22	0.28	<0.001
LA1	31.845223	-91.952627	16	16	0.17	0.058
AL1	31.906589	-87.38093	15	29	0.40	<0.001
AL2	33.280556	-87.406111	15	7	--	--
AR1	34.321154	-94.228551	6	15	0.31	0.014
SC1	34.42974	-81.113965	14	11	0.50	0.009
SC2	34.456535	-81.394315	16	11	0.79	<0.001
AL3	34.475076	-86.059406	16	20	0.29	<0.001
AR2	34.523745	-93.394406	15	16	0.39	0.003
TN1	35.090217	-87.47153	14	17	0.39	<0.001
TN2	35.181394	-84.441701	14	10	-0.01	1.000
NC1	35.200961	-81.666277	13	18	0.26	<0.001

Table 1 continued

TN3	35.874107	-87.449359	15	37	0.51	<0.001
AR3	35.996063	-92.210655	15	28	0.20	0.001
AR4	36.0281	-93.173925	15	33	0.27	<0.001
AR5	36.042529	-93.488241	16	13	-0.19	0.584
AR6	36.17592	-93.560042	15	11	0.57	0.011
NC2	36.344509	-79.206383	16	37	0.36	<0.001
TN4	36.480242	-84.662962	10	38	0.25	<0.001
TN5	36.524432	-84.888568	13	29	0.49	<0.001
MO1	36.91777	-92.08333	15	16	0.17	0.028
MO2	37.379804	-90.498671	15	32	0.54	<0.001
KY1	37.428524	-83.921127	16	29	0.55	<0.001
MO3	38.144548	-91.801243	15	21	0.78	<0.001
KY2	38.357575	-83.111572	16	45	0.18	<0.001
VA1	38.626929	-78.341875	16	21	0.41	<0.001
IN1	39.887444	-87.199419	16	33	0.56	<0.001
MI1	41.910382	-86.602865	16	51	0.12	0.006
IA1	41.992924	-93.886753	16	18	0.58	<0.001
MI2	42.650139	-83.558865	16	19	0.62	<0.001
WI1	44.530657	-90.070826	15	29	-0.04	0.337
WI2	45.141646	-87.194302	14	41	0.18	0.006
MN1	45.83063	-94.664244	16	22	0.41	<0.001
WI3	46.620762	-91.608392	15	31	0.33	<0.001

Table 1 continued

MN2	47.185761	-95.186577	16	24	0.35	<0.001
MN3	47.214236	-93.055252	16	37	0.42	<0.001

Table 2. Analysis of molecular variance within *Dirca palustris* based on microsatellite genotypes of 579 individuals from 39 populations. All F-statistics were significant at $P < 0.001$, except for the comparison of groups east and west of the Mississippi River.

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variance	Fixation index*
Populations					
Among populations	38	3231.84	5.40	52.04	0.52
Within populations	540	2685.66	4.97	47.96	
Two groups from STRUCTURE					
Among regions	1	300.04	0.78	7.24	0.07
Among populations	37	2931.80	5.01	46.53	0.50
Within populations	540	2685.66	4.97	46.23	0.54
East vs. west of Appalachian/ Apalachicola barrier					
Among regions	1	247.14	0.78	7.14	0.07
Among populations	37	2984.70	5.11	47.04	0.51
Within populations	540	2685.66	4.97	45.82	0.54
East vs. west of Mississippi River					
Among regions	1	129.60	0.18	1.72	0.02
Among populations	37	3102.24	5.32	50.78	0.52
Within populations	540	2685.66	4.97	47.51	0.53

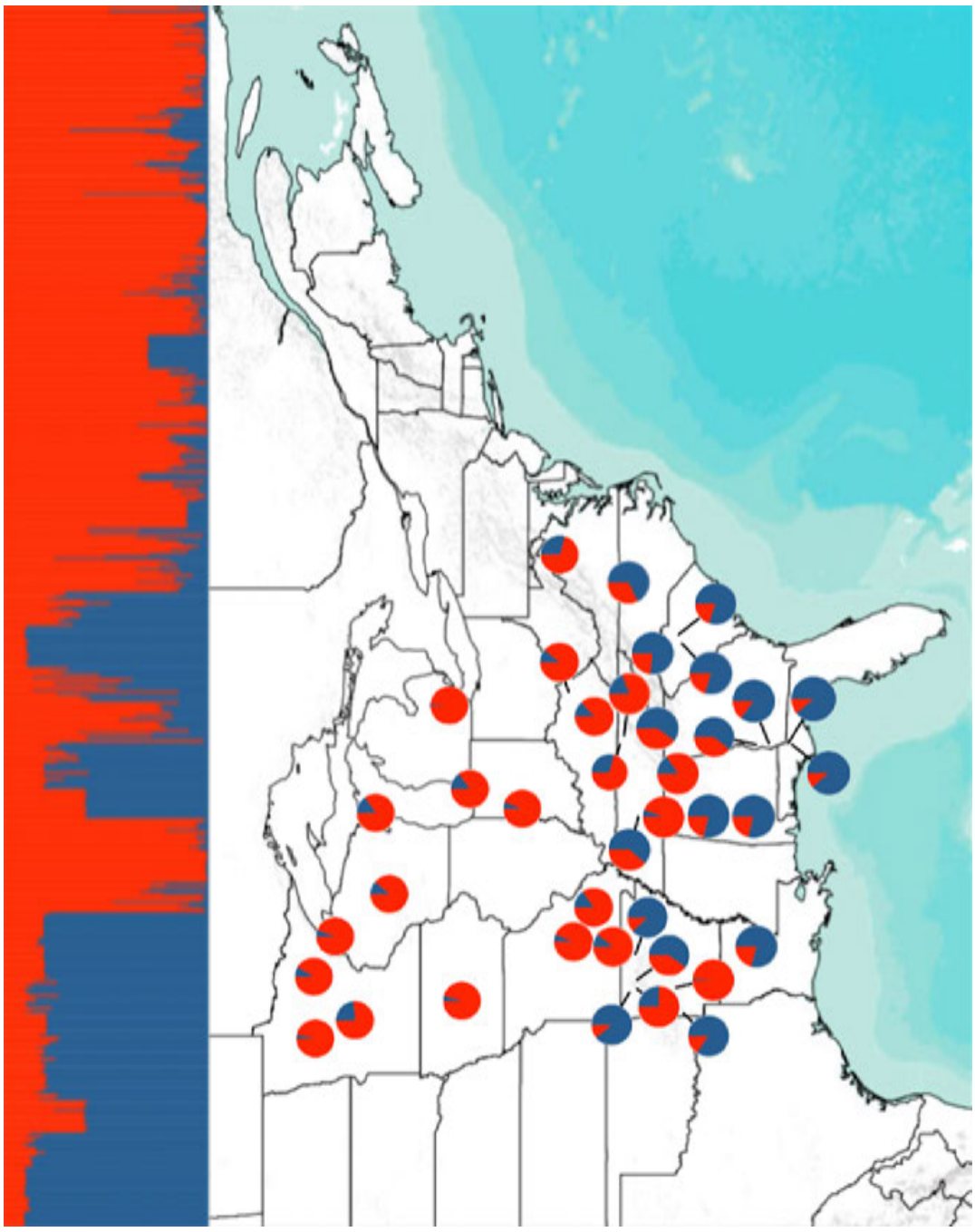

Fig. 1.

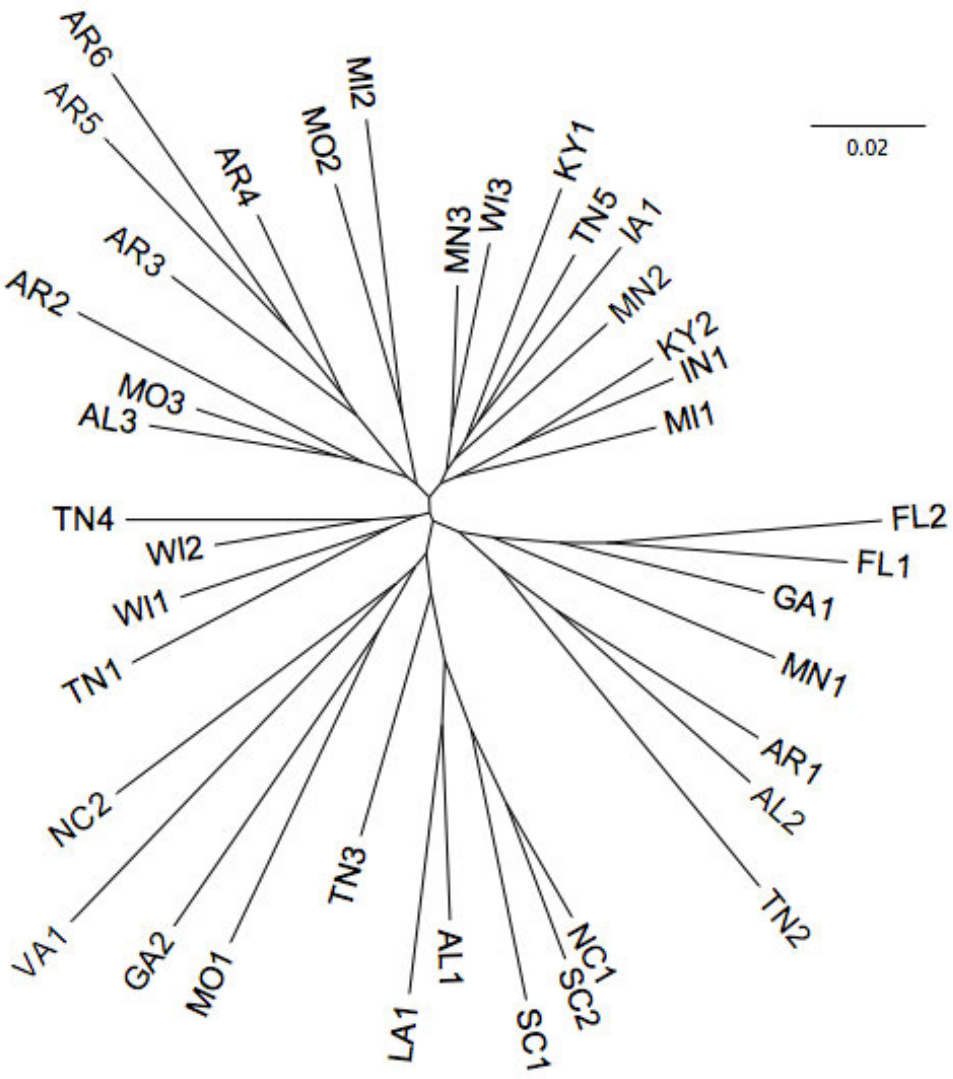
AR6
AR5
AR4
AR3
AR2
MO3
AL3
MI2
MO2
MN3
WI3
KY1
TN5
IA1
MN2
KY2
IN1
MI1
0.02
TN4
WI2
WI1
TN1
NC2
VA1
GA2
MO1
TN3
LA1
AL1
SC1
SC2
NC1
TN2
AL2
AR1
MN1
GA1
FL1
FL2

Fig. 2.

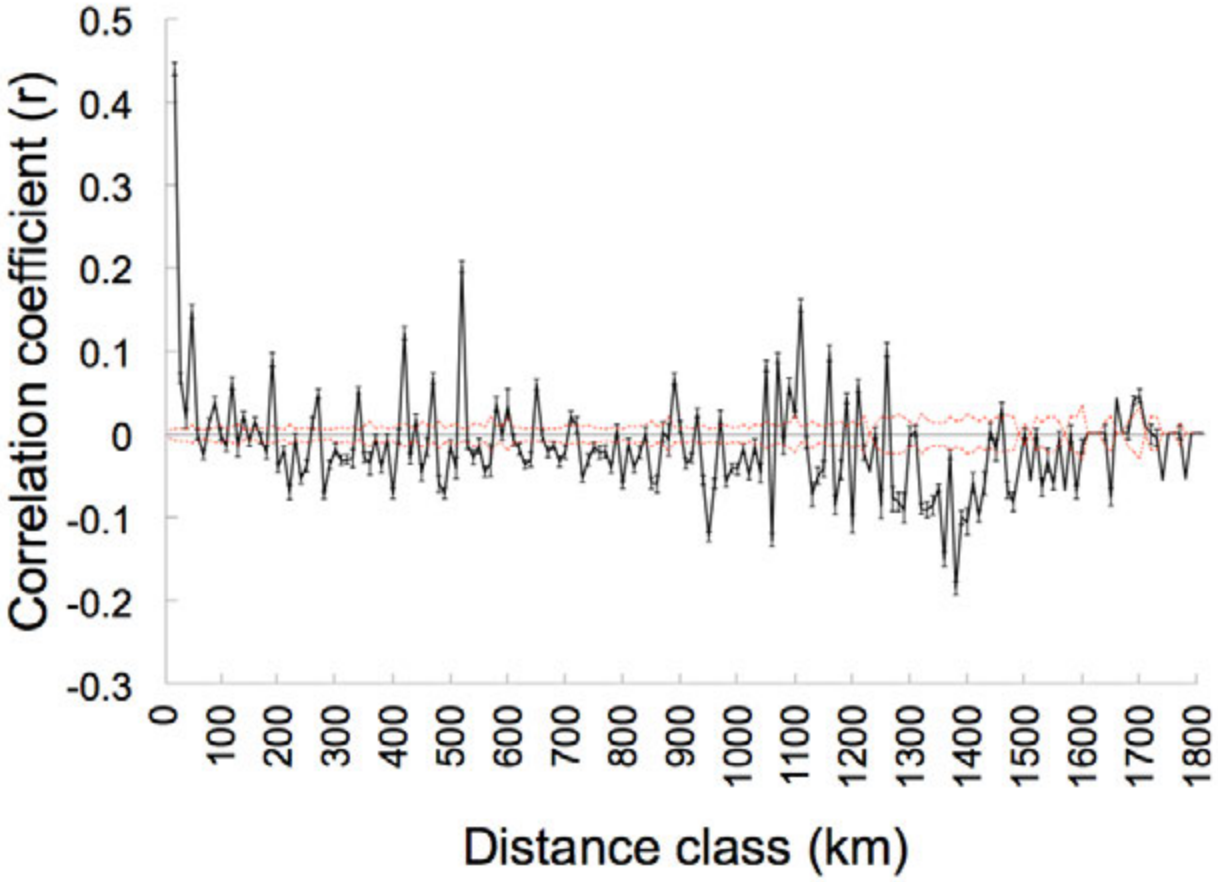
Correlation coefficient (r)
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
Distance class (km)

Figure 3.

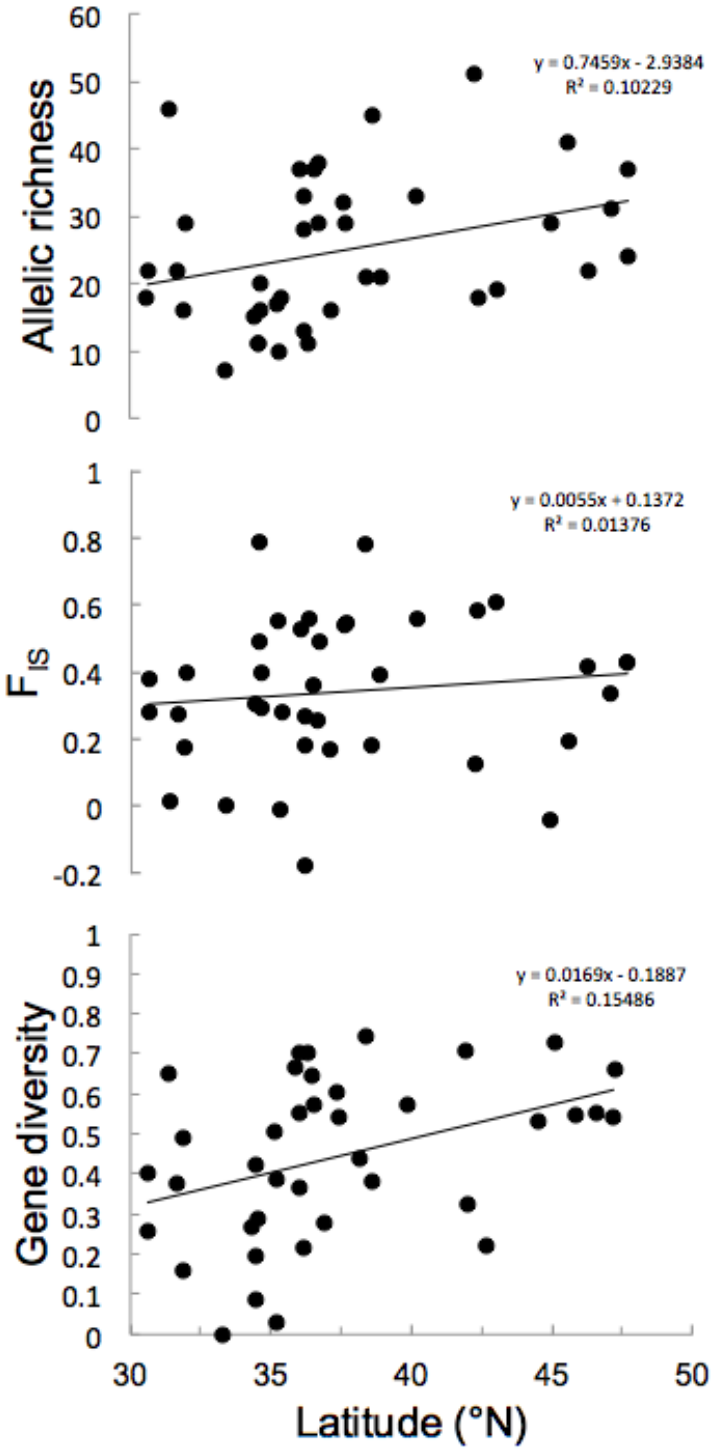
Allelic richness
y = 0.7459x - 2.9384
R² = 0.10229
F_IS
y = 0.0055x + 0.1372
R² = 0.01376
Gene diversity
y = 0.0169x - 0.1887
R² = 0.15486
Latitude (°N)

Fig. 4.

Figure Captions

Fig. 1. Results of STRUCTURE analysis of microsatellite allele frequencies from 39 populations (579 plants) of *Dirca palustris*. The bar chart indicates the probability that each plant belongs to either the southern (blue) or northern (red) genetic cluster. Individual plants are grouped by sampling sites, which are ranked by latitude from southernmost (left) to northernmost (right). The pie chart for each population shows the probability that it belongs to each cluster.

Fig. 2. Unrooted neighbor-joining dendrogram of genetic relationships among populations of *Dirca palustris*. Bootstrap values were low; the only values >50% were for the branch joining FL1 and FL2 (53%), the branch joining KY2 and IN1 (53%), and the branch joining SC2 and NC1 (60%).

Fig. 3. Spatial autocorrelation of genetic diversity across 39 populations of *D. palustris*. Values on the x-axis represent the maximum distance for the given 10-km distance class. The solid black line is the estimated correlation coefficient (r) for each distance class, which is a measure of pairwise genetic similarity among individuals separated by distances within that class. Dashed lines represent 95% confidence limits for the null hypothesis of no spatial structure.

Fig. 4. Correlations between latitude and allelic richness, F_{IS}, and gene diversity. Each circle represents one of 39 populations.

CHAPTER 5. EVIDENCE FOR A NEW SUBSPECIES OF *DIRCA PALUSTRIS* L. FROM THE SOUTHEASTERN UNITED STATES

Abstract

Evidence is presented for the recognition of a new subspecies, *Dirca palustris* L. subspecies *nivea* Peterson & Graves, from two populations in the panhandle of Florida, where the species is at its southernmost occurrence, and four additional populations throughout the southeastern U.S., where introgression among conspecifics apparently has occurred. Plants of the putative subspecies are characterized by white pubescence on involucral bracts rather than the brown described for the species, and calyx tubes that are distinctly shorter and with wider limbs than is typical for *D. palustris*. Populations in Florida share a distinct chloroplast haplotype based on five non-coding chloroplast regions, and microsatellite genetic comparisons among *D. palustris* further support a subspecies designation. Four additional populations, including one in Georgia, two in Alabama, and one in North Carolina, share the chloroplast haplotype and comprise plants with phenotypic characters similar to those of the plants in Florida. However, the four populations outside of Florida show greater phenotypic variation within populations, and greater genetic similarity to other populations of *D. palustris*, indicative of introgression between subspecies. Plants of the putative subspecies do not evidently differ morphologically in other ways from *D. palustris*, and the absence of reproductive barriers indicates that species-level designation is not warranted for plants of the southeastern populations we describe.

Introduction

Dirca L. comprises four named species of shrubs indigenous to North America. *Dirca palustris* L., which inhabits rich, mesic forest understories throughout eastern North America, was the first to be named (Gray 1868; Mottiar 2012). *Dirca occidentalis* Gray, endemic to varied habitats around the San Francisco Bay, was described based on numerous differences from *D. palustris* (Gray 1873; Vogelmann 1953). *Dirca mexicana* Nesom & Mayfield was described from a single population in northeastern Mexico (Nesom and Mayfield 1995), and *Dirca decipiens* Floden & Mayfield was named from several populations on the western edge of the range of *D. palustris* (Floden et al. 2009). At the times of their discoveries, the two latter species were regarded initially as *D. palustris* (F.G. Medrano, ASU specimen image ASU0050618; Freeman et al. 1998), but further systematic evaluations led to distinct species designations.

Although broadly distributed, *D. palustris* occurs sporadically across the landscape and often is absent from apparently suitable habitats. Dispersal limitation likely influenced the contemporary distribution of this species because fruits typically fall to the ground and become incorporated into the leaf litter beneath maternal plants (Ward and Horn 1998; Peterson and Graves 2011). Although fruits are produced annually, with little inter-annual variation in fruit abundance at the population level (Schulz et al. 2004), sexual recruitment can be low. Peterson and Graves (2011) documented particularly high predation of seeds, as well as little evidence for sexual recruitment over a six-year period in the southernmost population of *D. palustris,* in Liberty Co., Florida, where the species is listed as state-endangered (Wunderlin and Hansen 2008). Across the southern portion of its distribution, where the species is probably a Pleistocene relict (Delcourt and Delcourt 1975; Floden et al. 2009), populations are few and

highly localized. *Dirca palustris* is more common in northern hardwood forests of the Upper Great Lakes Region of northern Wisconsin and the Upper Peninsula of Michigan, where it sometimes is the dominant member of the shrub community (Schulz et al. 2004). Because of its abundance at higher latitudes and scarcity in the South, the species is especially vulnerable in southern populations.

Keys for identifying species of *Dirca* indicate that *D. palustris* can be distinguished from its congeners by brown pubescence on involucral bracts, pedicillate flowers and fruits, and glabrous to nearly glabrous leaves and stems (Vogelmann 1953; Nesom and Mayfield, 1995; Floden et al. 2009). We identified several populations in the southeastern portion of the range of *D. palustris* that exhibit stark white pubescence on involucral bracts, in contrast to the brown pubescence described for the species (Peterson et al. 2009; 2011). Shortly after, we studied the chloroplast phylogeography of *D. palustris* and found four additional populations with white-pubescent involucral bracts during visits to populations throughout eastern North America (Peterson 2013). Populations of white-pubescent plants share a distinct chloroplast haplotype that displays several mutational differences from other haplotypes across the range of *D. palustris* (Peterson 2013).

Because of our observations of plants in several populations in the Southeast, where the species may be vulnerable, we conducted a phenotypic and molecular-genetic evaluation of plants across the range of *D. palustris* to clarify the appropriate taxonomic rank of these unique Southeastern populations.

Materials and Methods

Phenotypic measurements

In 2011, we collected and pressed 306 specimens from 102 populations across the range of *D. palustris* to compare leaf and stem morphology. One voucher specimen from each population was deposited at ISC, and we intend to distribute duplicates to A, MO, NA, and NCU (Thiers 2013). All collections for this purpose were made after termination of annual stem elongation and leaf expansion. Measurements of leaf and stem morphology included stem elongation of the current and preceding year; length, width, and length:width of the three terminal-most leaves of the primary stem; and the presence of pubescence on the primary stem that elongated the year of collection and on both surfaces of the terminal leaf. T-tests with Satterthwaite correction for unequal variances were conducted in Microsoft Excel 2011 v.14.3.8 (Microsoft Corp., Redmond, WA) to test for differences in quantitative vegetative traits between the populations of white-pubescent plants and populations elsewhere.

In spring of 2012, we collected and pressed 126 specimens from 42 populations across the southern half of the range of *D. palustris* to record the color of pubescence on involucral bracts during anthesis. One voucher specimen from each population was deposited at ISC, and we intend to distribute duplicates to MO and NCU (Thiers 2013). Nine flowers, three from each of three inflorescences that were collected from each population and preserved in ethanol, were measured by using imageJ (Rasband 2012) on photographs taken under a stereomicroscope. Measurements of floral morphology included total calyx length, width of the calyx limb, and length:width of the calyx. Color of pubescence on involucral bracts was assessed as white, light brown, or dark brown based on subjective assessment with light brown representing any plant for which pubescence on involucral bracts was not white but was pale or

tan. T-tests assuming unequal variances were conducted to test for differences in quantitative floral traits between the populations with the southeastern haplotype and populations elsewhere.

Genetic analysis

Seven loci were amplified and genotyped from 579 individuals in 39 populations, following steps described in Peterson (2013). Populations included in the analyses are the same as those included in Peterson (2013). We used GenAlEx 6.1 (Peakall and Smouse 2006) to conduct an analysis of molecular variance (AMOVA) between the putative subspecies and plants elsewhere. Genetic data were available from four of the six populations with white pubescence on involucral bracts, including both populations in Florida (Torreya State Park, Liberty Co.; Aspalaga Landing, Gadsen Co.), a population in Alabama (Gullett's Bluff State Park, Wilcox Co.), and a population in North Carolina (Broad River south of Boiling Springs, Cleveland Co.). We compared results of analyses in which either all four genotyped populations of the putative subspecies, or only those in Florida, were compared with plants elsewhere.

Results

Phenotypic measurements

Several phenotypic traits separated plants of the putative subspecies from other *D. palustris*. Populations of the putative subspecies in Florida seemed to be fixed genetically for white pubescence on involucral bracts, as white pubescence was observed on all plants we encountered. Populations of the putative subspecies in Alabama, Georgia, and North Carolina were almost exclusively white, but several plants (ca. 5% of observed plants) had light-brown

pubescence on involucral bracts. In contrast, pubescence on involucral bracts of plants in other populations was dark to light brown (Table 1). In several populations of brown-pubescent plants in Alabama, South Carolina, and Tennessee, we found a low frequency of white-pubescent or nearly white-pubescent plants (Table 1), but with no evident geographical pattern in incidence.

A second phenotypic characteristic separating plants of the putative subspecies from plants elsewhere was the length and width of calyxes, which were shorter and had wider limbs on plants of the putative subspecies than among plants elsewhere (Table 1). As with color of pubescence on involucral bracts, plants in Florida were more absolute in their distinction from other *D. palustris* than were plants of the four other populations of the putative subspecies. Except for stem elongation, which averaged greater among plants of the putative subspecies, vegetative traits did not differ between plants of the putative subspecies and plants of *D. palustris* elsewhere (Table 2).

Genetic analysis

The four populations of white-pubescent plants that we evaluated with microsatellite genetic markers differed genetically from other populations of *D. palustris* (Table 3). AMOVA revealed that 4.2% of total genetic variation was partitioned between groups comprising the four populations of white-pubescent plants and plants elsewhere. However, when only plants from Florida were compared with plants elsewhere, 11.6% of total genetic variation was partitioned between groups.

Discussion

The phenotypic and genetic variation between plants of the distinct haplotype in the Southeast and plants elsewhere in the range of *D. palustris* warrants subspecies designation (Tables 1, 3). Within populations we studied in Florida, the apparently fixed trait of white pubescence on involucral bracts, the consistently shorter and broader calyx tubes, and the genetic differentiation from plants elsewhere demonstrate a clear and identifiable suite of minor characters suitable to differentiate subspecies. However, because color of pubescence on involucral bracts of plants in populations of the putative subspecies outside of Florida are occasionally light brown, and plants in those populations have slightly longer and more narrow calyx tubes than those from Florida (Table 1), we suspect introgression has occurred. Indeed, populations of typical *D. palustris* occur within several km of each of the populations for which we suspect some degree of introgression. The putative absence of reproductive barriers indicates that species-level designation is not warranted for plants of the southeastern phenotype.

It is likely that plants of *D. palustris* on bluffs of the Apalachicola River are Pleistocene relicts; they also are populations for which little contemporary exchange of genes has occurred with other populations. This region of the Florida panhandle is known for at least two other local endemics, *Torreya taxifolia* Am. and *Taxus floridana* Nutt. ex Chapm. Both are critically endangered and found only on the bluffs east of the Apalachicola River, narrowly sympatric with the putative subspecies of *D. palustris* in the region. This narrow endemism of other taxa co-occurring with the putative subspecies in Florida suggests white-pubescent populations of *D. palustris* in Florida long have occupied bluffs of this river system among a small cohort of other relicts, probably since before the Last Glacial Maximum.

Below is a short key to differentiate the putative subspecies, *D. palustris* subspecies *nivea,* from the broadly distributed typical subspecies and its congeners. This putative subspecies is identified from six locations throughout the southeastern U.S. (Table 4). The following key descriptions are from Floden et al. (2009) with minimal alterations for consistency.

1. Inflorescences on elongating peduncles, projecting out of the involucral bracts; calyx unlobed, the margin crenate, undulate, or erose; leaves usually glabrous, rarely somewhat uniformly pubescent

    2. Adaxial surface of involucral bracts with dark brown, rarely light brown tomentum; calyx length ≥ 2.5 times limb width ***D. palustris* ssp. *palustris***

    2. Adaxial surface of involucral bracts with white tomentum; calyx length < 2.5 times limb width ***D. palustris* ssp. *nivea***

1. Inflorescences remaining essentially sessile, glomerulate within the involucral bracts; adaxial surface of involucral bracts with white to light tan tomentum; calyx mostly 4-lobed, the margin entire to crenate; leaves and stems always uniformly pubescent to tomentose ***D. occidentalis, D. mexicana*, and *D. decipiens***

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Table 1. Color of pubescence on involucral bracts and floral measurements in *Dirca palustris* (126 plants from 42 populations). *P*-values from two-tailed t-tests comparing plants of the proposed subspecies to other plants of *D. palustris* were <0.05, as were *P*-values from the comparison of plants from Florida with other plants from the proposed subspecies.

Taxon	Involucral-bract pubescence[Z]	Calyx length (mm)	Limb width (mm)	Calyx length:width
D. palustris – proposed ssp.[Y]	W	6.2 (FL = 5.9)	2.7 (FL = 2.8)	2.4 (FL = 2.1)
D. palustris -- elsewhere	DB-LB (W)	6.7	2.2	3.1

[Z]Color of pubescence on involucral bracts ranged from white (W) to light brown (LB) to dark brown (DB). Letters in parentheses represent rare observations (<5% of specimens inspected).

[Y]Values are from all six populations of the proposed subspecies; values in parentheses include measurements from Florida populations alone.

Table 2. Phenotypic traits of leaves and stems from 305 specimens of *D. palustris* collected from 103 populations across its distribution. *P*-value from a two-tailed t-test comparing plants of the proposed subspecies to other plants of *D. palustris* was <0.05 for stem elongation, but not for leaf length or width.

Taxon	Leaf length (cm)[Z]	Leaf width (cm)[Z]	Petiole[Y]	Midrib[Y]	Blade[Y]	Stem[Y]	Stem (cm)
D. palustris – proposed ssp.[X]	8.0 (5.2-10.2)	4.8 (3.2-6.5)	G	G	G(P)	G	8.0
D. palustris – elsewhere	7.5 (2.7-11.7)	4.3 (1.5-7.9)	G(DP)	G(DP)	G(P)	G(P)	5.1

[Z]Length and width of three most terminal leaves on the primary stem. For all specimens, expansion of leaves and stems had terminated before the date of collection. Values in parentheses are the single least and greatest measurements recorded.

[Y]Presence or absence of pubescence on leaf petiole and abaxial midrib and blade, and on the stem that formed the year specimen was collected; G = glabrous; P = pubescent; DP = densely pubescent. Letters in parentheses represent rare observations (<5% of specimens inspected).

[X]Specimens were examined from four populations with the Southeastern haplotype (FL, AL, GA, NC).

Table 3. Analysis of molecular variance within *Dirca palustris* based on microsatellite genotypes of 579 individuals from 39 populations. All F-statistics were significant at $P < 0.001$.

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variance	Fixation index*
Proposed subspecies (FL) vs. others					
Among regions	1	164.99	1.34	11.57	0.12
Among populations	37	3066.86	5.26	45.44	0.51
Within populations	540	2685.66	4.97	42.99	0.57
Proposed subspecies (FL, AL, NC) vs. others					
Among regions	1	131.30	0.45	4.23	0.04
Among populations	37	3100.54	5.31	49.54	0.52
Within populations	540	2685.66	4.97	46.30	0.54

Table 4. Locations of the putative subspecies, *Dirca palustris* ssp. *nivea* Peterson & Graves.

Location	County	State	Latitude	Longitude
Torreya State Park	Liberty	Florida	30.590637	-84.935303
Aspalaga Landing	Gadsen	Florida	30.626095	-84.902344
Sanborn Creek	Decatur	Georgia	30.766582	-84.644959
Foster Creek	Henry	Alabama	31.360830	-85.111100
Gullett's Bluff State Park	Wilcox	Alabama	31.906589	-87.380930
Broad River	Cleveland	North Carolina	35.200961	-81.666277

GENERAL CONCLUSIONS

Resolving Reid's Paradox

The contemporary distribution of eastern leatherwood raises fascinating phylogeographical questions. How did the species endure the rapid changes in climate during late-Pleistocene climate oscillations? Where did it persist during the Last Glacial Maximum? By what phenomena did seeds disperse to the north following the retreat of the continental ice sheets? What is the relationship between scattered, relictual populations in the South and larger, more robust populations to the north? It is with questions like these that the search for clues begins in earnest, with the recognition that we probably can never know the answers with certainty. But the quest for often-elusive answers is driven not just by the grip that the natural world has on us, but also by pressing concerns and challenges facing us today.

One of our urgent issues in the 21st Century is the question of what effects climate change will have on our landscape. Phylogeographers recognize that if we hope to predict what the future holds, we ought to understand the past more fully. We need to gain understanding wherever we can, with whatever evidence we can find and test, of the broadest pictures of the distant past and of the intricacies (and challenges) now unfolding before us. It is for these reasons that I studied the phylogeography of *Dirca*. I conducted this work not because I believed that investigating the history of *D. palustris* would provide us a complete picture, but because there is always so much left to learn, and one has to start somewhere.

In this dissertation, I evaluated Reid's paradox of plant migration as it relates to the contemporary distribution of *D. palustris*, a shrub that has a patchy distribution and no known agents for long-distance dispersal, yet extends to the northernmost reaches of the eastern United

States and into Canada. I considered alternative resolutions to this paradox. Perhaps the species persisted further north than traditionally has been suggested by those studying species distributions in the past. Perhaps distances of dispersal routinely exceeded those expected from contemporary observations. Possibly, the full picture has some combination of the two.

I conclude that *D. palustris* likely persisted at latitudes further north than the traditionally cited Gulf-Coastal refugia for temperate forest species. In fact, the species plausibly existed within several hundred kilometers of the Laurentide ice sheet. This conclusion is based on the collective results of ecological niche modeling and the distribution of chloroplast haplotypes and genetic variation in microsatellite loci of *D. palustris*. The plausibility of mid-latitudinal refugia of *D. palustris*, and indeed many temperate forest species, is difficult to deny given the results of this work and that of other investigators.

What should we make of the alternative hypothesis, that dispersal simply exceeded contemporary estimates of the capacity for plant migration? For *D. palustris*, both hypotheses seem to have merit. My work revealed an exceptional diversity of chloroplast haplotypes at northern latitudes and a peculiar maintenance of genetic diversity with northward migration. When we consider the genetic evidence for occasional long-distance dispersal, it is clear that dispersal of *D. palustris* during the current interglacial exceeded intuitive estimates based on contemporary observations. But what accounts for this? Within populations, spatial clustering of plants is common, and fruits fall to the ground beneath maternal plants. Even when we account for the presence of refugia at middle latitudes during the Last Glacial Maximum, an average rate of dispersal of tens of meters per year would be necessary to account for the northward range limits of *D. palustris*, a species with no known tendency for long-distance dispersal.

The implications of this last point are not trivial. Either *D. palustris* routinely disperses further than our collective observations would suggest, or there once existed an agent in the forests of eastern North America that facilitated dispersal of the species. Both conclusions beg for a change to the way we look at the security of our eastern forests, and the responses they might have to a future with changing climates. Although any explanation I put forward on this point risks being received as a mere just-so story, I find that one suggestion in particular is worth mentioning. The passenger pigeon, once the most abundant bird in the world, no doubt influenced the demographic and genetic structure of eastern North American forests. How could it not? With numbers in the billions, it cleared nuts and other seeds from the understories of large swaths of forest during annual migrations. In their travels from south to north, passenger pigeons certainly carried a variety of viable seeds in their crops. It is not unreasonable to expect that seeds of numerous taxa occasionally escaped digestion, either by regurgitation, a behavior that was common among passenger pigeons when more nutritious sustenance was encountered, or by the abrupt death of the bird, a common outcome of predation.

Additional Outcomes

Aside from my findings on the phylogeography of *D. palustris*, this research had two additional outcomes. The first was a demonstration of the feasibility of *de novo* isolation of highly polymorphic microsatellites by next-generation sequencing from a nonmodel woody plant without prior enrichment of genomic DNA for microsatellite repeats. The approach proved to be rapid and economical, and further economy was obtained throughout screening loci and genotyping individuals with fluorescently tagged universal primers and multiplexing the amplification products. Indeed, it seems that microsatellite genetics projects, even those

conducted on plants for which no prior genomic information is available, can be accomplished by researchers with limited budgets and basic knowledge of molecular genetics.

The final outcome of this work is the discovery of a putative subspecies, *Dirca palustris* L. ssp. *nivea* Peterson & Graves, from the southeastern United States. Some populations in the region share a distinct chloroplast haplotype, white pubescence on involucral bracts, and calyces that are shorter and have wider limbs than are typical for the species. The conservation value of these populations should be assessed, as populations in the Southeast are scarce and restricted to cool, moist niches.

Future Work

Future work within the genus *Dirca* could involve additional studies to clarify fine-scale genetic structure, perhaps with a more focused effort on the Great Lakes Region, where plants were necessarily absent during the Last Glacial Maximum. Analyses capable of explicit tests of alternative hypotheses about the colonization of previously glaciated landscapes would be of tremendous value to phylogeography. There is also much work to be done with numerous other plant taxa of eastern North America. Few, if any, shrubs of eastern North America have been evaluated from a phylogeographical perspective, and many other components of the eastern forests have yet to be investigated. It is my hope that with an ever-growing body of literature on the phylogeography of eastern North America, we can come to a more complete understanding of the past and future composition of our natural landscapes.

Interpretive Summary

Changes in climate threaten the survival of many plant species. The forests of eastern North America contain many species that may face extinction unless they can migrate to regions with favorable climates. I studied responses of a shrub, eastern leatherwood (*Dirca palustris*), to changing climates during the past 20,000 years. During this time, North America transformed from a much cooler continent with massive sheets of ice over Canada and the northern United States during the Last Glacial Maximum to the warmer continent we have today. I modeled suitable climate for eastern leatherwood and projected it onto climate conditions estimated for 20,000 years ago to determine where the species might have existed. I collected DNA from plants across the range of eastern leatherwood and analyzed variation among individuals to determine relationships. Climate modeling for the Last Glacial Maximum showed that the species could have existed far north of the Gulf Coast, where scientists traditionally believed temperate forest species persisted. Genetic evidence suggested that plants of eastern leatherwood occurred within 500 km (~300 miles) of the ice sheets during the Last Glacial Maximum, in present-day Tennessee, Kentucky, Arkansas, and Missouri. My results also indicated very limited spread of the species by seeds on a local scale but unexplained long-distance spread to the Upper Great Lakes Region, which was previously covered by ice. Finally, I found evidence that certain eastern leatherwoods in the southeastern United States should be renamed as a subspecies. My results add to our collective knowledge of where plant species existed in the past, how their ranges expanded as climates changed, and how plants might respond to changing climates in the future.

APPENDIX. HERBARIUM VOUCHER LABELS FOR COLLECTIONS OF *DIRCA PALUSTRIS* L.

This appendix includes one voucher label from each population visited during the course of dissertation research. Although multiple specimens were collected from each population, the list beginning on the following page includes only one label per population for brevity.

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Grundy Co., **MISSOURI**.
Coordinates: 40.118802, -93.682319

Near top of north-facing sandstone bluffs of ravine tributary to Thompson River, ca. 4 miles NW of Trenton. Undeveloped property of Crowder State Park. Accessibe from the south by following Equestrian Trailhead at northern end of NW Dove Lane, then walking through old pasture.

Additional taxa at site: *Acer saccharum, Quercus rubra, Juglans nigra, Ostrya virginiana, Zanthoxylum americanum.*

Bryan J. Peterson: 2011-1A

May 19, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Maries Co., **MISSOURI**.
Coordinates: 38.144548, -91.801243

Spring Creek Gap Conservation Area; several dozen plants in floodplain of Cedar Creek and slopes of ravine leading to it. Soil of floodplain is very sandy. Accessed by parking at Spring Creek Gap parking area and following small tributary stream in bottom of ravine.

Additional taxa at site: *Acer saccharum, Ostrya virginiana, Quercus, Aesculus glabra, Fraxinus, Cornus florida.*

Bryan J. Peterson: 2011-2A

May 21, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Boone Co., **MISSOURI**.
Coordinates: 38.758913, -92.204664

West-facing slope east of Ashland Lake; several dozen plants found uphill from edge of floodplain, with several plants in floodplain.

Additional taxa at site: *Acer saccharum, Quercus rubra, Juglans nigra, Ostrya virginiana, Zanthoxylum americanum.*

Bryan J. Peterson: 2011-3A

May 21, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Howell Co., **MISSOURI**.
Coordinates: 36.9122, -92.09229

Northeast of Noblett Lake; several dozen plants along trails northeast of Noblett Lake picnic grounds, above Noblett Creek.

Bryan J. Peterson: 2011-4A

May 22, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Wright Co., **MISSOURI**.
Coordinates: 37.093629, -92.679548

Cedar Gap Conservation Area; several dozen plants along headwaters to Bryant Creek and on slopes above creek. Plants are present within sight of the old cabin near the creek. Follow trail from parking area down to creek; trail criss-crosses creek several times, after which leatherwood plants will be visible sporadically from the trail.

Additional taxa at site: *Acer saccharum, Carpinus caroliniana, Staphylea trifolia, Aesculus glabra, Lindera benzoin, Asimina triloba, Cornus florida, Platanus occidentalis, Quercus rubra, Ostrya virginiana.*

Bryan J. Peterson: 2011-5A

May 22, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Scott Co., **TENNESSEE**.
Coordinates: 36.480242, -84.662962

Large population at Leatherwood Crossing; hundreds of plants along trail south of Leatherwood Ford parking area. Several plants found at edge of parking lot.

Bryan J. Peterson: 2011-6A
with Aaron Floden

May 26, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

McCreary Co., **KENTUCKY**.
Coordinates: 36.629985, -84.72697

Great Meadow Campground; several dozen plants along trail through floodplain of Rock Creek.

Additional taxa at site: *Acer saccharum, Fagus grandifolia, Carpinus caroliniana, Tsuga canadensis, Aesculus glabra, Lindera benzoin, Asimina triloba, Tilia americana, Liquidambar styraciflua, Liriodendron tulipifera, Acer rubrum.*

Bryan J. Peterson: 2011-7A
with Aaron Floden

May 26, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Campbell Co., **TENNESSEE**.
Coordinates: 36.32483, -84.08337

About a dozen plants on south-facing slope of creek in Whitman Hollow, south of bend in Low Gap Road. Private property.

Additional taxa at site: *Acer saccharum, Liriodendron tulipifera, Asimina triloba, Lindera benzoin, Thuja occidentalis, Fraxinus pennsylvanica, Tilia americana.*

Bryan J. Peterson: 2011-8A
with Aaron Floden

May 26, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Polk Co., **TENNESSEE**.
Coordinates: 35.181394, -84.441701

Locally common along north shore of Hiawassee River; many dozens of plants found between Powerhouse Road and River on the way to the Dam.

Bryan J. Peterson: 2011-9A

May 27, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Claiborne Co., **TENNESSEE**.
Coordinates: 36.540041, -83.631561

Steep north-facing slope above Powell River; several dozen plants on wooded slope west of Cumberland Gap Parkway (Interstate 25E).

Bryan J. Peterson: 2011-10A
with Aaron Floden

May 27, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Grainger Co., **TENNESSEE**.
Coordinates: 36.402149, -83.45614

Just east of Interstate 25E bridge over Clinch River; several dozen plants near east end of gravel road on north- and east-facing slopes along south shore of river.

Bryan J. Peterson: 2011-11A
with Aaron Floden

May 27, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Hickman Co., **TENNESSEE**.
Coordinates: 35.874107, -87.449359

Locally common along Bell Branch; many dozens of plants just east of bridge on Old Mill Creek Road, along floodplain between creek and road.

Additional taxa at site: *Acer saccharum, Magnolia tripetala, Liquidambar styraciflua, Platanus occidentalis, Sassafras albidum, Lindera benzoin.*

Bryan J. Peterson: 2011-12A

May 28, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Newton Co., **ARKANSAS**.
Coordinates: 36.0208, -93.35371

Ponca Landing; plants locally common on north- to northeast-facing slopes above Leatherwood Creek; several hundred plants of various ages present at site.

Bryan J. Peterson: 2011-13A

May 29, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Wayne Co., **MISSOURI**.
Coordinates: 37.260802, -90.504502

Sam A. Baker State Park; dozens of plants in floodplain woods north of Big Creek.

Bryan J. Peterson: 2011-14A

May 29, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Madison Co., **MISSOURI**.
Coordinates: 37.379804, -90.498671

Near unincorporated town of Jewett. Dozens of plants located along Country Road 435 southeast of bridge where State Hwy. C crosses Leatherwood Creek. Quite common along creeks and slopes of the countryside in the area.

Bryan J. Peterson: 2011-15A

May 29, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Newton Co., **ARKANSAS**.
Coordinates: 36.0281, -93.173925

Northeast of Jasper; small stream north of hairpin turn in Hwy. 7 and west of gravel road; dozens of plants on east- to northeast- facing slope above stream.

Bryan J. Peterson: 2011-16A

May 29, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Reynolds Co., **MISSOURI**.
Coordinates: 37.56381, -90.92513

Woods east of the Middle Fork of the Black River; at least a dozens plants. Parking spot on side of road. Property ownership unknown.

Additional taxa at site: *Acer saccharum, Carpinus caroliniana, Aesculus, Quercus alba, Juglans nigra, Platanus occidentalis, Carya ovata, Fraxinus quadrangulata, Asimina triloba, Gleditsia triacanthos.*

Bryan J. Peterson: 2011-17A

May 29, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca decipiens Floden & Mayfield

Johnson Co., **KANSAS**.
Coordinates: 38.795838, -94.691634

Natural area of Overland Park Arboretum; locally common on north-facing slope south of Wolf Creek; hundreds of plants visible from trail.

Additional taxa at site: *Quercus, Cercis canadensis, Carya, Celtis occidentalis, Asimina triloba, Juglans nigra, Ulmus, Staphylea trifolia, Corylus.*

Bryan J. Peterson: 2011-18A

June 2, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca decipiens Floden & Mayfield

Carroll Co., **ARKANSAS**.
Coordinates: 36.465955, -93.760285

Locally common along small stream north of Nightingale Road at intersection with Hwy. 187 and in floodplain east of highway; hundreds of plants seen, mostly east of highway near Leatherwood Creek. Property east of highway had a "For Sale" sign by it.

Additional taxa at site: *Acer saccharum, Asimina triloba, Prunus, Carpinus caroliniana, Lindera benzoin, Quercus, Juniperus, Ulmus, Aesculus, Staphylea trifolia.*

Bryan J. Peterson: 2011-19A

June 3, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Madison Co., **ARKANSAS**.
Coordinates: 35.89543, -93.58223

Along trail to King's River Falls on the King's River; several plants spotted on rocky shores within the first several hundred yards from County Road 3500.

Additional taxa at site: *Acer saccharum, Ulmus, Carpinus caroliniana, Lindera benzoin, Staphylea trifolia, Alnus, Hamamelis, Physocarpus opulifolius, Rhamnus caroliniana.*

Bryan J. Peterson: 2011-20A

June 3, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca decipiens Floden & Mayfield

Carroll Co., **ARKANSAS**.
Coordinates: 36.169167, -93.544759

Less than a dozen plants located near bridge at sharp bend in County Road 524 (locally called Dripping Springs Road); several plants uphill and several plants dowhill from road. Location on very steep slopes above the Dry Fork of Kings River make plants difficult to access. Several plants formerly at the edge of the road were destroyed by county brush mower.

Bryan J. Peterson: 2011-21A
with Larry Lowman

June 4, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Carroll Co., **ARKANSAS**.
Coordinates: 36.17592, -93.560042

Wooded slopes south above Dry Fork of the Kings River; many dozens of plants located in shallow ravine just southeast of intersection between County Road 524 and County Road 546. Several plants are accessible from right-of-way, with many more on private property and requiring permission from landowner to access.

Bryan J. Peterson: 2011-22A
with Larry Lowman

June 4, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Stone Co., **ARKANSAS**.
Coordinates: 35.991827, -92.209711

Gunner Pool Recreation Area; dozens of plants on north- and east-facing slopes high above North Sylamore Creek. Accessed by taking the North Sylamore Creek Trail, which crosses Gunner Pool Road near the sign at the entrance to the recreation area.

Bryan J. Peterson: 2011-23A

June 5, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Monroe Co., **ALABAMA**.
Coordinates: 31.546393, -87.515631

Locally common on slopes south of bridge at Claiborne Landing; dozens of plants are accessible by parking on the road under the bridge and crossing through bamboo and steep slope to small ravine below.

Additional taxa at site: *Acer saccharum, Cladrastis kentukea, Aesculus, Carpinus caroliniana, Fagus grandifolia, Magnolia, Ilex.*

Bryan J. Peterson: 2011-24A

June 6, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Wayne Co., **MISSISSIPPI**.
Coordinates: 31.83022, -88.52882

Three plants on south shore of Shiloh Creek east of Waynesboro Matherville Road bridge.

Additional taxa at site: *Ostrya virginiana, Carpinus caroliniana, Hamamelis, Aesculus, Fagus grandifolia.*

Bryan J. Peterson: 2011-26A

June 6, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Oregon Co., **MISSOURI**.
Coordinates: 36.787188, -91.34516

Greer Spring, Mark Twain National Forest; about a dozen plants scattered along creek near spring outlet. Access from trailhead on west side of Hwy. 19.

Bryan J. Peterson: 2011-27A

June 6, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Franklin Co., **KENTUCKY**.
Coordinates: 38.287377, -84.860158

Steeles Branch tributary to Kentucky River; several dozen plants on north- and east-facing slopes above Steele Branch Road.

Bryan J. Peterson: 2011-28A

June 10, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Breckinridge Co., **KENTUCKY**.
Coordinates: 37.81728, -86.292909

Dent's Bridge Rosetta Road; several dozen plants along road above Sinking Creek. Some plants are adjacent to the road, while others are on slope over creek.

Bryan J. Peterson: 2011-29A

June 12, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Hardin Co., **KENTUCKY**.
Coordinates: 37.716313, -85.74758

Cap Hollow; dozens of plants on north- to northwest-facing slope above small, possibly seasonal, tributary to Younger Creek. Access by following trail at curve in Miller Road, southeast of open grassy picnic area.

Additional taxa at site: *Acer saccharum, Fagus grandifolia, Staphylea trifolia, Aesculus, Hydrangea, Lindera benzoin, Asimina triloba, Prunus, Hamamelis, Cornus, Carpinus caroliniana*

Bryan J. Peterson: 2011-30A

June 12, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Webster Co., **IOWA**.
Coordinates: 42.420193, -94.102235

Woodman Hollow State Preserve; dozens of plants located on steep, north-facing slopes above creek tributary to Des Moines River. Crossing to south side of creek is treacherous and steep slopes can be muddy. Access to preserve is from small grass parking area off Woodmans Hollow Road.

Additional taxa at site: *Acer saccharum, Tilia americana, Quercus alba, Quercus rubra, Carya, Ulmus, Fraxinus, Prunus.*

Bryan J. Peterson: 2011-31A
with Andrew Chase

June 15, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Boone Co., **IOWA**.
Coordinates: 41.992924, -93.886753

Ledges State Park; several dozen plants located on north-facing slopes of Reindeer Ridge. Plants accessible by trail system overlooking the stream. Several plants at Table Rock and many more on steep slopes over stream.

Additional taxa at site: *Acer saccharum, Quercus alba, Quercus rubra, Carpinus caroliniana, Fraxinus, Ostrya virginiana, Ulmus, Tilia americana, Staphylea trifolia, Prunus.*

Bryan J. Peterson: 2011-32A

June 16, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Ramsey Co., **MINNESOTA**.
Coordinates: 44.942142, -93.197236

Shadow Falls Ravine east of the Mississippi River; dozens of plants on steep north- to northwest- faving slope of ravine. Located based on an historical record from the 1910's.

Additional taxa at site: *Acer saccharum, Populus deltoides, Tilia americana, Cornus, Carpinus caroliniana, Ostrya virginiana.*

Bryan J. Peterson: 2011-33A
with Emilie Justen

June 23, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Vernon Co., **WISCONSIN**.
Coordinates: 43.690068, -90.795502

Northwestern part of Jersey Valley County Park; several hundred plants on north-facing slopes above seasonal streams. Plants are common on north-facing slopes elsewhere in the park. Evidence of herbivore damage on stems.

Additional taxa at site: *Acer saccharum, Fraxinus, Ostrya virginiana, Populus tremuloides, Tilia americana.*

Bryan J. Peterson: 2011-34A

June 24, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Olmsted Co., **MINNESOTA**.
Coordinates: 44.101436, -92.135822

Whitewater State Wildlife Management Area; dozens of plants on steep north-facing slope above North Fork of the Whitewater River. Accessed by parking on 72nd Street NE near abandoned building and hiking to slopes above river. Evidnece of herbivore damage on stems. Additional group of plants found at 44.101802, -92.131909.

Additional taxa at site: *Acer saccharum, Pinus strobus, Viburnum dentatum, Staphylea trifolia, Ostrya virginiana, Populus, Cornus, Prunus, Tilia americana, Carpinus caroliniana, Xanthoxylum americanum, Quercus alba.*

Bryan J. Peterson: 2011-35A

June 24, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Marathon Co., **WISCONSIN**.
Coordinates: 44.902855, -90.221892

Cherokee Park; several dozen plants on gentle north-facing slopes above Big Eau Pleine River. Follow Indian Trail west from parking area.

Additional taxa at site: *Acer saccharum, Fraxinus, Tsuga canadensis, Carpinus caroliniana, Prunus, Ostrya virginiana, Tilia americana, Betula alleghaniensis, Picea glauca.*

Bryan J. Peterson: 2011-36A
with Alisha Peterson

June 25, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Taylor Co., **WISCONSIN**.
Coordinates: 45.121018, -90.61213

Chequamegon-Nicolet National Forest; several dozen plants in open woods beside trail, all fairly small. Did not scout for additional plants.

Additional taxa at site: *Acer saccharum, Fraxinus, Populus tremuloides, Tilia americana, Viburnum, Corylus cornuta, Prunus, Carpinus caroliniana.*

Bryan J. Peterson: 2011-37A
with Alisha Peterson

June 25, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Wood Co., **WISCONSIN**.
Coordinates: 44.530657, -90.070826

North-facing slopes of Power's Bluff County Park; several dozen plants in woods east of skiing/tubing hill and in wooded strips between runs.

Bryan J. Peterson: 2011-38A
with Alisha Peterson

June 25, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Grant Co., **WISCONSIN**.
Coordinates: 43.014849, -90.758853

Big Green River; several dozen plants in highly localized pouplation on north-facing slope above river and Green River Road.

Additional taxa at site: *Acer saccharum, Aesculus glabra, Staphylea trifolia, Carya, Fraxinus, Juniperus virginiana, Carpinus caroliniana, Cornus, Tilia americana.*

Bryan J. Peterson: 2011-39A

June 26, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Cavalier Co., **NORTH DAKOTA**.
Coordinates: 48.964722, -98.100278

West of Little Pembina River; extremely fecund site with many hundreds of plants both on flat terrain and north-facing slopes near seasonal streams tributary to Little Pembina River gorge. Sandy soil with shale-like deposits. Reasonably accessible by parking at the 90-degree turn in gravel road (intersection of 107th Street and 118th Ave).

Additional taxa at site: *Quercus macrocarpa, Acer negundo, Fraxinus, Prunus, Viburnum dentatum, Cornus sericea, Corylus.*

Bryan J. Peterson: 2011-40A
collected by Bill Graves

June 26, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Clayton Co., **IOWA**.
Coordinates: 42.813584, -91.340343

Southeast of Elkader, Rentz Memorial Woods State Preserve; several dozen plants scattered in ravine and on ridge north of Grain Road. Much herbivore damage to plants.

Additional taxa at site: *Staphylea trifolia, Zanthoxylum americanum, Acer saccharum, Ostrya virginiana, Juniperus virginiana, Prunus, Carya ovata, Quercus alba, Fraxinus, Carpinus caroliniana.*

Bryan J. Peterson: 2011-41A

June 27, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Clayton Co., **IOWA**.
Coordinates: 42.678397, -91.39801

Bixby State Park; wooded slope on trail near ice cave. Only one plant observed, about five to six feet tall.

Bryan J. Peterson: 2011-42A

June 27, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Hardin Co., **IOWA**.
Coordinates: 42.445251, -93.09221

South shore of Iowa River in Leverton Timber; many dozens of plants on slope above river. Not easy to access. Plants might be on private property adjacent to Leverton Timber; location of property boundary unclear. Much damage to plants by herbivore browsing; many leatherwoods are very short and carpet the ground.

Additional taxa at site: *Tilia americana, Staphylea trifolia, Carpinus caroliniana, Ostrya virginiana, Zanthoxylum americanum, Acer saccharum, Quercus alba, Ribes, Fraxinus, Celtis occidentalis, Betula, Prunus.*

Bryan J. Peterson: 2011-43A

June 28, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Barry Co., **MISSOURI**.
Coordinates: 36.579048, -93.835477

Several plants growing by drainage along Eagle's Nest Trail, just southwest of the campground located south of the Roaring River.

Bryan J. Peterson: 2011-44A

July 2, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Catahoulah Parish Co., **LOUISIANA**.
Coordinates: 31.845223, -91.952627

Plants locally abundant in shallow ravine of a small tributary to Sugar Creek, hundreds of plants just northest of intersection between road and gas pipeline right-of-way.

Additional taxa at site: *Fagus grandifolia, Magnolia, Pinus, Asimina triloba, Aesculus pavia, Acer saccharum, Ilex, Viburnum dentatum, Carya.*

Bryan J. Peterson: 2011-45A

July 4, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Howard Co., **ARKANSAS**.
Coordinates: 34.321154, -94.228551

On floodplain of Cassatot River near Cassatot Falls; several dozen plants between driveway and shore, with some plants uphill from driveway near picnic areas.

Additional taxa at site: *Asimina triloba, Ilex, Quercus rubra, Fraxinus quadrangulata, Carpinus caroliniana, Ostrya virginiana, Cercis canadensis, Quercus alba, Pinus, Acer saccharum, Symphoricarpos orbiculatus.*

Bryan J. Peterson: 2011-46A

July 4, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Garland Co., **ARKANSAS**.
Coordinates: 34.523745, -93.394406

Plants located along Murphy Creek and seasonal tributaries off Albert Pike Road (Hwy. 270); several dozen plants near creek on south side of road and in drainage northeast of the bridge.

Additional taxa at site: *Liquidambar styraciflua, Pinus taeda, Ilex opaca, Asimina triloba, LIndera benzoin, Cornus florida, Vitis rotundifolia, Quercus nigra, Carya, Acer rubrum, Nyssa sylvatica.*

Bryan J. Peterson: 2011-47A

July 4, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Wilcox Co., **ALABAMA**.
Coordinates: 31.906589, -87.38093

Small population on north-facing slope at Gullet's Bluff; ca. two dozen plants on rolling terrain east of wide trail originating at parking area.

Additional taxa at site: *Ostrya virginiana, Acer saccharum, Acer rubrum, Ilex, Aesculus, Carya ovata, Liriodendron tulipifera, Hamamelis, Carpinus caroliniana, Platanus occidentalis.*

Bryan J. Peterson: 2011-48A

July 8, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Tuscaloosa Co., **ALABAMA**.
Coordinates: 33.280556, -87.406111

Small population on slopes of ravine tributary to Black Warrior River; ca. two dozen plants on slopes beside old gravel road leading to river. Easiest access is to follow old gravel road from north side of Rocky Branch Road.

Additional taxa at site: *Acer saccharum, Aesculus, Fagus grandifolia, Vitis, Hydrangea quercifolia, Carpinus caroliniana, Tilia americana, Carya ovata, Magnolia tripetala, Ostrya virginiana, Quercus, Cercis canadensis, Cladrastic kentukea, Hamamelis.*

Bryan J. Peterson: 2011-49A

July 8, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Marshall, Dekalb Co., **ALABAMA**.
Coordinates: 34.475076, -86.059406

South side of Sauty Creek, Buck's Pocket State Park; plants scattered sporadically on north-facing slopes above creek. Plants are scarce and on very difficult terrain east of the campground. Plants west of the primary concrete bridge crossing Sauty Creek are located uphill from the TVA road, and also locally common in a portion of floodplain.

Additional taxa at site: *Liquidambar styraciflua, Pinus taeda, Ilex opaca, Asimina triloba, LIndera benzoin, Cornus florida, Vitis rotundifolia, Quercus nigra, Carya, Acer rubrum, Nyssa sylvatica.*

Bryan J. Peterson: 2011-50A

July 9, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Colbert Co., **ALABAMA**.
Coordinates: 34.622508, -87.794323

Plants located within Cane Creek Canyon Nature Preserve; ocassionally scattered along streams within the preserve.

Additional taxa at site: *Liquidambar styraciflua, Pinus taeda, Ilex opaca, Asimina triloba, LIndera benzoin, Cornus florida, Vitis rotundifolia, Quercus nigra, Carya, Acer rubrum, Nyssa sylvatica.*

Bryan J. Peterson: 2011-51A
with Jim Lacefield

July 9, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Lawrence Co., **TENNESSEE**.
Coordinates: 35.090217, -87.47153

Plants located along south side of Clack Branch on north side of Clax Branch Road; several dozen plants found among garbage dumped along the road.

Additional taxa at site: *Liquidambar styraciflua, Pinus taeda, Ilex opaca, Asimina triloba, LIndera benzoin, Cornus florida, Vitis rotundifolia, Quercus nigra, Carya, Acer rubrum, Nyssa sylvatica.*

Bryan J. Peterson: 2011-52A

July 9, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Winston Co., **ALABAMNA**.
Coordinates: 34.28301, -87.39558

South shore of Sipsey River, William Bankhead National Forest; several plants on sandy shore east of Cranal Road crossing.

Additional taxa at site: *Carpinus caroliniana, Lindera benzoin, Asimina triloba, Hydrangea quercifolia, Acer saccharum, Euonymus americanus, Cercis canadensis, Ilex, Pinus, Cornus, Tsuga canadensis.*

Bryan J. Peterson: 2011-53A

July 9, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Clay Co., **GEORGIA**.
Coordinates: 31.616136, -85.049303

Several dozen plants located on north-facing slope of ravine over Town Branch in Fort Gaines. Terrain is steep and difficult, with old trash dumping evident.

Bryan J. Peterson: 2011-54A
collected by Bill Graves

July 10, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Liberty Co., **FLORIDA**.
Coordinates: 30.590637, -84.935303

Northeastern part of Torreya State Park; several hundred plants locally common on rolling terrain near seasonal streams south of Apalachicola River. Plants are accessible by traversing steep slopes below foot path.

Additional taxa at site: *Fagus grandifolia, Liquidambar styraciflua, Liriodendron tulipifera, Magnolia grandiflora, Pinus glabra, Ostrya virginiana, Ilex opaca, Carya, Acer saccharum.*

Bryan J. Peterson: 2011-55A
collected by Bill Graves

July 11, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Gadsen Co., **FLORIDA**.
Coordinates: 30.626095, -84.902344

On slopes east of Apalachicola River; dozens of plants localized to limestone outcroppings near the parking area.

Additional taxa at site: *Carpinus caroliniana, Aesculus, Platanus occidentalis, Acer saccharum.*

Bryan J. Peterson: 2011-56A
collected by Bill Graves

July 11, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Newberry Co., **SOUTH CAROLINA**.
Coordinates: 34.407852, -81.449153

Several plants in a ravine of the stream valley tributary to the Enoree River at the end of Sumter National Forest Road 1086 north of County Route 55. Access to plants is somewhat difficult.

Additional taxa at site: *Liquidambar styraciflua, Pinus taeda, Ilex opaca, Asimina triloba, LIndera benzoin, Cornus florida, Vitis rotundifolia, Quercus nigra, Carya, Acer rubrum, Nyssa sylvatica.*

Bryan J. Peterson: 2011-57A
with Charles Horn

July 13, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Fairfield Co., **SOUTH CAROLINA**.
Coordinates: 34.42974, -81.113965

Locally common along Minton Creek; hundreds of plants in floodplain of creek on both sides of gravel road. To access site, follow forestry road located north of railroad tracks off Old Chester's Road, keeping right past two forks and finding plants where road crosses over a large culvert.

Additional taxa at site: *Liquidambar styraciflua, Pinus taeda, Ilex opaca, Asimina triloba, LIndera benzoin, Cornus florida, Vitis rotundifolia, Quercus nigra, Carya, Acer rubrum, Nyssa sylvatica.*

Bryan J. Peterson: 2011-58A
with Charles Horn

July 13, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Fairfield Co., **SOUTH CAROLINA**.
Coordinates: 34.4565357, -81.394315

South shore of Beaver creek at bridge crossing of County Route 99; several dozen plants along creek on both sides of road.

Additional taxa at site: *Liquidambar styraciflua, Pinus taeda, Ilex opaca, Asimina triloba, LIndera benzoin, Cornus florida, Vitis rotundifolia, Quercus nigra, Carya, Acer rubrum, Nyssa sylvatica.*

Bryan J. Peterson: 2011-59A
with Charles Horn

July 13, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Aiken Co., **SOUTH CAROLINA**.
Coordinates: 33.542327, -81.995834

Along southern banks of Fox Creek; dozens of plants scattered west/southwest of intersection of Bergen Road and West Martintown Road (Hwy. 230).

Additional taxa at site: *Acer saccharum, Carpinus caroliniana, Ostrya virginiana, Cornus, Liriodendron tulipifera, Quercus, Parthenocissus quinquefolia.*

Bryan J. Peterson: 2011-60A

July 14, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Cleveland Co., **NORTH CAROLINA**.
Coordinates: 35.200961, -81.666277

South of Boiling Springs; hundreds of plants along southern shore of Broad River, west of Gaffney Road (Hwy. 150) bridge. No plants found east of bridge.

Additional taxa at site: *Acer rubrum, Acer saccharum, Pinus, Cornus, Asimina triloba, Ilex opaca, Carya, Carpinus caroliniana, Liquidambar styraciflua, Liriodendron tulipifera, Sassafras albidum, Quercus.*

Bryan J. Peterson: 2011-61A

July 16, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Laurel Co., **KENTUCKY**.
Coordinates: 37.174197, -84.280901

Steep north-facing slopes above Rockcastle River; several dozen plants sporadically distributed between Hawk Creek Road and the river below.

Additional taxa at site: *Lindera benzoin, Asimina triloba, Fagus grandifolia, Aesculus, Acer saccharum, Carya, Hamamelis, Magnolia tripetala, Fraxinus, Tsuga canadensis, Liriodendron tulipifera, Carpinus caroliniana, Cercis canadensis.*

Bryan J. Peterson: 2011-62A

July 17, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Jackson Co., **KENTUCKY**.
Coordinates: 37.428524, -83.921127

Turkey Foot Recreation Area; several dozen plants sporadically scattered along south shore of War Fork. Accessible by several trails from the grassy picnic area at the curve in Girl Scout Road.

Additional taxa at site: *Acer sacccharum, Lindera benzoin, Staphylea trifolia, Magnolia tripetala, Ulmus, Fagus grandifolia, Hamamelis virginiana, Parthenocissus quinquefolia, Aesculus, Cornus, Cercis canadensis, Liriodendron tulipifera, Asimina triloba, and Juglans nigra.*

Bryan J. Peterson: 2011-63A

July 17, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Breathitt Co., **KENTUCKY**.
Coordinates: 37.45874, -83.16401

Slope along entrance to Robinson Forest; about a dozen plants along Clemens Fork Road on slope above Clemons Fork.

Additional taxa at site: *Acer saccharum, Fraxinus, Lindera benzoin, Magnolia, Asimina triloba, Fagus grandifolia, Quercus, Liriodendron tulipifera, Platanus occidentalis, Juglans nigra, Carpinus caroliniana, Tsuga canadensis.*

Bryan J. Peterson: 2011-64A

July 17, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Adair Co., **KENTUCKY**.
Coordinates: 37.010886, -85.498683

East-northeast of Edmonton; about a dozen plants along small creek just northeast from intersection of William Judd Road and Columbia/Edmonton Road. Small stream appears to be tributary to nearby Leatherwood Creek.

Additional taxa at site: *Carpinus caroliniana, Asimina triloba, Lindera benzoin, Fagus grandifolia, Fraxinus, Aesculus, Platanus occidentalis, Juniperus virginiana, Quercus, Carya, Ulmus, Vitis.*

Bryan J. Peterson: 2011-65A

July 21, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Summers Co., **WEST VIRGINIA**.
Coordinates: 37.719659, -80.890493

Camp Brookside south of town of Brooks; abundant in understory of woods on east side of New River. Accessible by parking at boat landing; turn south off Temple Street (Hwy 20), drive across railroad tracks, turn left onto rough gravel road, and follow to boat landing. Trail from landing to woods close to shore.

Additional taxa at site: *Acer saccharum, Fraxinus, Quercus rubra, Chionanthus virginicus, Juniperus virginiana, Cercis canadensis, Carya, Ulmus, Rhus, Carpinus caroliniana, Rosa, Quercus alba.*

Bryan J. Peterson: 2011-66A

July 22, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Carter Co., **KENTUCKY**.
Coordinates: 38.357575, -83.111572

Cascade Caves State Park; several dozen plants on north-facing slope above Tygarts Creek and in ravine north of Cascade Road (Hwy. 209). Accessible by parking on north side of Cascade Road and following trails down slope.

Additional taxa at site: *Cornus, Hamamelis, Asimina triloba, Staphylea trifolia, Quercus, Ostrya virginiana, Acer saccharum, Viburnum, Ulmus, Vitis, Rhus aromatica, Cercis canadenis, Fraxinus, Tsuga canadensis, Fagus grandifolia.*

Bryan J. Peterson: 2011-67A

July 22, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Caswell Co., **NORTH CAROLINA**.
Coordinates: 36.344509, -79.206383

Caswell County Game Land; Dozens of plants on east-facing slopes above Hyco Creek. Most plants were less than 3' tall and were sporadically distributed over several acres.

Additional taxa at site: *Fagus grandifolia, Acer saccharum, Asimina triloba, Carya, Ostrya virginiana, Lindera benzoin, Vitis, Cornus.*

Bryan J. Peterson: 2011-68A

July 23, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Page Co., **VIRGINIA**.
Coordinates: 38.626929, -78.341875

Mill Prong east of Skyline Drive, Shenandoah National Park; several dozen plants along trail on south side of Mil Prong, and southeast of point at which trail crosses the creek. Accessible by parking at Milam Gap, following Appalachian Trail (white blazes) across Skyline Drive and taking the Mill Prong trail (blue blazes) to Mill Prong.

Additional taxa at site: *Lindera benzoin, Betula alleghaniensis, Tsuga canadensis, Hamamelis virginiana, Ostrya virginiana, Acer saccharum, Acer rubrum, Acer pensylvanicum.*

Bryan J. Peterson: 2011-69A

July 23, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Westchester Co., **NEW YORK**.
Coordinates: 41.210254, -73.561049

Halle Ravine, Pound Ridge; several dozen plants in bottom of ravine and on slopes over unnamed creek. Accessible by parking at entrance to trail system near Trinity Pass intersection with Burns/Danbrook Road. Forest duff layer is essentially absent; soil is light and fluffy.

Additional taxa at site: *Fagus grandifolia, Acer saccharum, Tsuga canadensis, Lindera benzoin, Euonymus, Ostrya viginiana, Tilia americana, Carpinus caroliniana.*

Bryan J. Peterson: 2011-70A

July 27, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Butler Co., **OHIO**.
Coordinates: 39.552765, -84.732571

Heuston Woods State Park; about a dozen plants in north- and northwest-facing ravine over Four Mile Creek south of Acton Lake. Accessible from parking area west of bridge; walk east across bridge and follow trail along east side of creek.

Bryan J. Peterson: 2011-71A

July 30, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Parke Co., **INDIANA**.
Coordinates: 39.887444, -87.199419

Newby Gulch, Turkey Run State Park; many dozens of plants in woods just northeast of Turkey Run Nature Center and on slopes west of Newby Gulch.

Additional taxa at site: *Acer saccharum, Fagus grandifolia, Lindera benzoin, Asimina triloba, Staphylea trifolia, Fraxinus, Quercus alba, Carpinus caroliniana, Cornus, Cercis canadensis, Prunus, Ribes, Ulmus.*

Bryan J. Peterson: 2011-72A

July 30, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

York Co., **NEW BRUNSWICK**.
Coordinates: 45.992183, -66.867933

Small population in woods on northeast-facing slope of Keswick Ridge. Private property, with gates and fences blocking access without permission.

Additional taxa at site: *Acer saccharum, Ostrya virginiana, Acer spicatum, Fagus grandifolia, Juglans cinerea, Thuja occidentalis, Abies balsamea, Betula populifolia, Betula alleghaniensis, Corylus cornuta.*

Bryan J. Peterson: 2011-73A
collected by Bill Graves

July 30, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Hants Co., **NOVA SCOTIA**.
Coordinates: 45.037833, -63.46845

Barney's Brook; several dozen plants onundulating topography of northeast-facing slope above brook. Accessible by parking at gate and walking less than half an hour up old road to cabin; plants immediately behind cabin.

Additional taxa at site: *Acer saccharum, Abies balsamea, Betula papyrifera, Ostrya virginiana, Tsuga canadensis, Picea glauca, Fraxinus americana, Acer spicatum, Fagus grandifolia.*

Bryan J. Peterson: 2011-74A
collected by Bill Graves

July 31, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Aroostook Co., **MAINE**.
Coordinates: 45.931283, -68.32

Small population of about a dozen plants in woods northeast of Sherman Mills.

Additional taxa at site: *Abies balsamea, Taxus canadensis, Fagus grandifolia, Ostrya virginiana, Fraxinus americana, Tilia americana, Acer saccharum.*

Bryan J. Peterson: 2011-75A
collected by Bill Graves

July 31, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Washington Co., **VERMONT**.
Coordinates: 44.2857, -72.4375

Taylor Farm Road just northwest of Plainfield; dozens of plants on both sides of road (mostly west) to at least 100 feet north of intersection with Vermont 214.

Additional taxa at site: *Acer saccharum, Abies balsamea, Pinus glauca, Fagus grandifolia, Fraxinus americana, Corylus.*

Bryan J. Peterson: 2011-76A
collected by Bill Graves

August 1, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Hampshire Co., **MASSACHUSETTS**.
Coordinates: 42.309917, -72.524217

Mount Holyoke Range State Park; population on east- to northeast-facing slope beside Brookbank Trail along Plum Creek. Northeast of Notch Visitors Center.

Additional taxa at site: *Ostrya virginiana, Fagus grandifolia, Fraxinus americana, Tsuga canadensis, Quercus, Hamamelis, Kalmia/Rhododendron.*

Bryan J. Peterson: 2011-77A
collected by Bill Graves

August 2, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Berkshire Co., **MASSACHUSETTS**.
Coordinates: 42.6722, -73.252883

In woods of Field Farm property; population of dozens of plants on gentle slope along Oak Loop trail following eastern edge of Field Farm property. Parking area is at south end of property on Sloan Road.

Additional taxa at site: *Acer saccharum, Fagus grandifolia, Carya cordiformis, Tilia americana, Fraxinus americana, Ostrya virginiana, Viburnum, Sambucus canadensis, Acer spicatum, Lindera benzoin.*

Bryan J. Peterson: 2011-78A
collected by Bill Graves

August 2, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Strafford Co., **NEW HAMPSHIRE**.
Coordinates: 43.229583, -71.071383

Woods at eastern base of Mount Misery cliffs and west of U.S. 202; several dozen plants among large rocks in botanically rich community on gentle east- to northeast-facing slope.

Additional taxa at site: *Ostrya virginiana, Acer saccharum, Betula alleghaniensis, Corylus, Hamamelis, Fraxinus americana, Quercus rubra.*

Bryan J. Peterson: 2011-79A
collected by Bill Graves

August 2, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Door Co., **WISCONSIN**.
Coordinates: 45.068982, -87.145228

Locally common behind Bailey's Harbor Cemetery; many dozens of plants on west-facing slope behind cemetery and on rolling terrain south of cemetery.

Additional taxa at site: *Acer saccharum, Tilia americana, Quercus rubra, Betula papyrifera, Fraxinus, Ostrya virginiana, Fagus grandifolia, Abies balsamea, Prunus.*

Bryan J. Peterson: 2011-80A
with Adam Peterson, Ulrike Zenner, Laura Peterson

August 22, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Door Co., **WISCONSIN**.
Coordinates: 45.141646, -87.194302

Common along Highland Road at eastern border of Peninsula State Park; several hundred plants in woods along both sides of road just north of Main Street.

Additional taxa at site: *Fagus grandifolia, Tsuga canadensis, Acer saccharum, Ostrya virginiana, Fraxinus, Picea alba, Tilia americana, Betula papyrifera.*

Bryan J. Peterson: 2011-81A
with Adam Peterson, Ulrike Zenner, Laura Peterson

August 23, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Alger Co., **MICHIGAN**.
Coordinates: 46.381584, -87.077062

Common in woods at Laughing Whitefish Falls Scenic Site; dozens to hundreds of plants on rolling terrain east of parking loop on Laughing Whitefish Falls Road.

Additional taxa at site: *Acer saccharum, Fagus grandifolia, Abies balsamea, Ostrya virginiana.*

Bryan J. Peterson: 2011-82A
with Adam Peterson, Ulrike Zenner, Laura Peterson

August 24, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Marquette Co., **MICHIGAN**.
Coordinates: 46.72553, -87.71442

Yellow Dog River; several dozen plants along County Road 510, about 500 feet south of bridge river. Found on both sides of road in uneven, rocky woods with sandy soil.

Additional taxa at site: *Acer saccharum, Betula alleghaniensis, Ostrya virginiana, Tsuga canadensis, Tilia americana, Acer rubrum, Abies balsamea, Fagus grandifolia, Acer pensylvanicum.*

Bryan J. Peterson: 2011-83A
with Adam Peterson, Ulrike Zenner, Laura Peterson

August 24, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Iron Co., **MICHIGAN**.
Coordinates: 46.094646, -88.429657

Common on northern edge of Bewabic State Park; many dozens to hundreds of plants in woods along Hwy. 2. Stems of plants are dark black instead of golden-brown more typically found in the species.

Additional taxa at site: *Acer saccharum, Ostrya virginiana, Fraxinus, Betula alleghaniensis, Tilia americana, Picea glauca.*

Bryan J. Peterson: 2011-84A
with Adam Peterson, Ulrike Zenner, Laura Peterson

August 25, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Gogebic Co., **MICHIGAN**.
Coordinates: 46.26378, -89.25743

Common in Sylvania Wilderness; hundreds of plants in woods south of NF-6320 east of Thousand Island Lake Road.

Additional taxa at site: *Acer saccharum, Betula papyrifera, Prunus, Abies balsamea, Tilia americana.*

Bryan J. Peterson: 2011-85A
with Adam Peterson, Ulrike Zenner, Laura Peterson

August 25, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dircа palustris L.

Marquette Co., **MICHIGAN**.
Coordinates: 46.277138, -88.035246

Along unnamed road south of Fence River Road leading to northern end of Twin Lakes; several dozen plants in rolling terrain of woods.

Additional taxa at site: *Acer saccharum, Quercus rubra, Abies balsamea, Betula papyrifera, Tsuga canadensis.*

Bryan J. Peterson: 2011-86A
with Adam Peterson, Ulrike Zenner, Laura Peterson

August 25, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dircа palustris L.

Potter Co., **PENNSYLVANIA**.
Coordinates: 41.482217, -77.73185

Large population along Italian Hollow Trail; hundreds of plants in and around seasonal stream tributary to Greenlick Run. Some plants exceeding 12' tall with trunk diameters exceeding 5". Many plants with dense, rounded form in somewhat sunny understory. Observed several ramets attached via rhizomes to parent ramets in eroded streambed. Herb layer dominated by ferns. Accessed by parking in small gravel loop off Greenlick Road and following trail to north/east.

Additional taxa at site: *Acer saccharum, Fagus grandifolia, and Ostrya virginiana.*

Bryan J. Peterson: 2011-87A

August 28, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dircа palustris L.

Forest Co., **PENNSYLVANIA**.
Coordinates: 41.536173, -79.4364355

Sibbald Run tributary to Allegheny River; several dozen plants located along small stream. Accessible from small parking spot along U.S. 62.

Additional taxa at site: *Acer saccharum, Carpinus caroliniana, Lindera benzoin, Ostrya virginiana, Carya, Platanus occidentalis, Fraxinus, Betula, Hamamelis.*

Bryan J. Peterson: 2011-88A

August 28, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dircа palustris L.

Blue Earth Co., **MINNESOTA**.
Coordinates: 44.157385, -93.988785

North-facing slope south of Glenwood Avenue in Mankato; several dozen plants, likely more.

Additional taxa at site: *Staphylea, Carya cordiformis, Prunus, Carpinus caroliniana, Fraxinus, Acer saccharum, Celtis occidentalis, Ostrya virginiana, Juglans nigra, Ribes, Tilia americana.*

Bryan J. Peterson: 2011-89A

September 9, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Nicollet Co., **MINNESOTA**.
Coordinates: 44.26686, -94.041325

Northeast- and northwest-facing slopes above Sevenmile Creek; dozens of plants on both sides of trail near top of slope. Accessible by walking west on trail from parking area and taking trail to left just before crossing second bridge.

Additional taxa at site: *Tilia americana, Quercus macrocarpa, Acer saccharum, Xanthoxylum americana, Ostrya virginiana, Amelanchier, Carya cordiformis, Quercus alba, Quercus rubra, Ribes, Celtis occidentalis, Viburnum dentatum.*

Bryan J. Peterson: 2011-90A

September 9, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Wright Co., **MINNESOTA**.
Coordinates: 45.312538, -94.10887

Southwest of Bass Lake; several dozen plants on rolling terrain along Knowles Avenue NW.

Additional taxa at site: *Acer saccharum, Tilia americana, Rosa, Ostrya virginiana, Quercus rubra, Carya.*

Bryan J. Peterson: 2011-91A

September 9, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Todd Co., **MINNESOTA**.
Coordinates: 45.83063, -94.664244

Northwest of Strump Lake, Oak Ridge State Wildlife Management Area; several dozen plants on north-facing slope just east of entry to management area from 341st Avenue.

Additional taxa at site: *Acer saccharum, Quercus rubra, Populus tremuloides, Xanthozylum americanum, Cornus alternifolia, Tilia americana, Ribes, Fraxinus, Prunus, Viburnum dentatum, Ostrya virginiana.*

Bryan J. Peterson: 2011-92A

September 10, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Otter Tail Co., **MINNESOTA**.
Coordinates: 46.699224, -95.786267

State Aquatic Management Area; several dozen plants on gentle east-facing wooded slope along W. Lake 7 Road by lagoon on the northwest corner of Scalp Lake. Additional plants visible on west side of road, but property is posted as private.

Additional taxa at site: *Acer saccharum, Tilia americana, Ostrya virginiana, Populus grandidentata, Ulmus, Fraxinus.*

Bryan J. Peterson: 2011-93A

September 10, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Clearwater Co., **MINNESOTA**.
Coordinates: 47.185761, -95.186577

Locally common within Itasca State Park; several hundred plants on rolling terrain in woods on both sides of trail to fire tower, between Jeanne Lake and Allen Lake. Parking for trail is off Wilderness Drive, a one-way loop around the park.

Additional taxa at site: *Acer saccharum, Acer rubrum, Ostrya virginiana, Viburnum dentatum, Cornus, Tilia americana, Quercus rubra, Corylus.*

Bryan J. Peterson: 2011-94A

September 10, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Polk Co., **MINNESOTA**.
Coordinates: 47.591872, -95.605576

Common within Hagen Wildlife Area; several hundred plants along trail from 405th Ave.

Additional taxa at site: *Acer saccharum, Ostrya virginiana, Quercus rubra, Tilia americana, Ribes, Viburnum dentatum, Fraxinus, Abies balsamea, Juglans nigra, Populus tremuloides, Prunus, Acer negundo, Zanthoxylum americanum, Quercus alba, Quercus macrocarpa.*

Bryan J. Peterson: 2011-95A

September 10, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Polk Co., **MINNESOTA**.
Coordinates: 47.61236, -96.050198

Abundant along western edge of Farmers Union Park, west of Lake Sarah; perhaps several hundred plants in woods to east of 200th Avenue. Some plants immediately adjacent to road ditch in nearly full sun.

Additional taxa at site: *Tilia americana, Acer saccharum, Ostrya virginiana, Quercus rubra, Zanthoxylum americanum, Ribes, Ulmus.*

Bryan J. Peterson: 2011-96A

September 10, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Douglas Co., **WISCONSIN**.
Coordinates: 46.620762, -91.608392

Brule River State Forest; dozens to hundreds of plants on rolling terrain in woods surrounding Tower at the end of Fire Tower Road.

Additional taxa at site: *Acer saccharum, Tilia americana, Ostrya virginiana, Abies balsamea, Betula papyrifera, Quercus rubra, Populus tremuloides.*

Bryan J. Peterson: 2011-97A

September 11, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

St. Louis Co., **MINNESOTA**.
Coordinates: 47.214236, -93.055252

Locally common on Beauty Mountain; several hundred plants seen on slopes above and below Beauty Mountain Road.

Additional taxa at site: *Acer saccharum, Betula alleghaniensis, Quercus rubra, Tilia americana, Ribes, Ostrya virginiana, Populus tremuloides, Viburnum, Abies balsamea, Picea glauca, Acer rubrum, Populus grandidentata.*

Bryan J. Peterson: 2011-98A

September 11, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

St. Louis Co., **MINNESOTA**.
Coordinates: 47.38368, -92.954793

Locally common on trail system to east of Tower Road on the way to summit of Maple Hill; several hundred plants in open, rocky understory. Sparse shrub layer almost exclusively Dirca.

Additional taxa at site: *Acer saccharum, Acer rubrum, Quercus rubra, Tilia americana, Betula papyrifera, Ostrya virginiana.*

Bryan J. Peterson: 2011-99A

September 11, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca mexicana Nesom & Mayfield

Story Co., **IOWA**.
Coordinates: 42.02708, -93.64473

Plants derived from seeds collected at the single known population of Dirca mexicana in Tamaulipas, Mexico (23.98583, -99.47694). Specimens collected from plants grown on campus of Iowa State University.

Bryan J. Peterson: 2011-100A
collected by Bill Graves

September 14, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Berrien Co., **MICHIGAN**.
Coordinates: 41.910382, -86.602865

Warren Dunes State Park; hundreds of plants scattered throughout woods over sand dunes. Dirca plants found almost immediately upon entering wooded trails just north of parking lot.

Additional taxa at site: *Acer saccharum, Quercus rubra, Tilia americana, Carpinus caroliniana, Prunus, Sassafras albidum, Hamamelis, Ostrya virginiana, Fraxinus, Asimina triloba, Lindera benzoin, Tsuga canadensis, Liriodendron tulipifera, Ptelea trifoliata.*

Bryan J. Peterson: 2011-101A

September 17, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Oakland Co., **MICHIGAN**.
Coordinates: 42.650139, -83.558865

Haven Hill, Highland Recreation Area; several hundred plants in damp woods along trail north of Haven Hill Lake. Trail accessible by parking southeast of the lake and crossing Ford Dam on the east.

Additional taxa at site: *Acer saccharum, Ostrya virginiana, Lindera benzoin, Fraxinus, Fagus grandifolia, Quercus, Prunus, Tilia americana, Carya.*

Bryan J. Peterson: 2011-102A

September 18, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Benzie Co., **MICHIGAN**.
Coordinates: 44.769155, -86.054513

Southern portion of Sleeping Bear Dunes National Lakeshore; several hundred plants in woods south of Aral Road between Aral Hills Road and Northland Hwy./Hwy. 22. Additional plants in small patch of woods northeast of the interstection of Norconk Road and Manning Road (44.778918, -86.057298).

Additional taxa at site: *Acer saccharum, Ostrya virginiana, Fraxinus, Tsuga canadensis, Tilia americana.*

Bryan J. Peterson: 2011-103A

September 18, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Cheboygan Co., **MICHIGAN**.
Coordinates: 45.485141, -84.685864

Common in Chaboiganing Nature Preserve; hundreds of plants on rolling terrain in understory of woods beside Lathers Road on peninsula west of Burt Lake.

Additional taxa at site: *Acer saccharum, Fagus grandifolia, Tilia americana, Tsuga canadensis, Betula papyrifera, Ostrya virginiana, Fraxinus, Quercus rubra, Ulmus, Juglans nigra, Vitis, Crataegus.*

Bryan J. Peterson: 2011-104A

September 18, 2011

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Henry Co., **ALABAMA**.
Coordinates: 31.360830, -85.111100

Plants scattered in woods east of intersection of George H. Grimsley Hwy. (Hwy. 95) and Foster Creek.

Bryan J. Peterson: 2012-4A
collected by Bill Graves

February 9, 2012

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Decatur Co., **GEORGIA**.
Coordinates: 30.766582, -84.644959

Plants growing on wooded slope at intersection of Duke Wells Road and Sanborn Creek, ca. 1.5 km north of Faceville.

Bryan J. Peterson: 2012-5A
collected by Bill Graves

February 10, 2012

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Early Co., **GEORGIA**.
Coordinates: 31.353875, -85.064258

Common on north-facing wooded slope along Freeman Creek at intersection with Old River Road; ca. 0.3 km north of intersection of Old River Road and Chancey Mill Road.

Bryan J. Peterson: 2012-54A
collected by Bill Graves

February 10, 2012

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Wilcox Co., **ALABAMA**.
Coordinates: 31.818367, -87.384789

Small population on norhtwest-facing slope tributary to Tallatchee Creek; several dozen plants on slope to west side of road, just south of Pine Flat Cemetery and church on east side.

Additional taxa at site: *Fagus grandifolia, Vaccinium, Ostrya virginiana, Pinus, Magnolia, Ilex, Rhododendron, Quercus, Liriodendron.*

Bryan J. Peterson: 2012-10A
with Laura Peterson

February 13, 2012

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Cherokee Co., **SOUTH CAROLINA**.
Coordinates: 35.026860, -81.490241

Abundant in woods along Broad River; many dozens to hundreds of plants on relatively flat terrain west of boat landing at the end of Ninety-nine Ferry Road, southeast of dam.

Bryan J. Peterson: 2012-21A

March 18, 2012

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Fentress Co., **TENNESSEE**.
Coordinates: 36.524430, -84.888570

Occasional on wooded north-facing slope above Pogue Creek and Williams Creek Road.

Bryan J. Peterson: 2012-25A

March 19, 2012

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Claiborne Co., **TENNESSEE**.
Coordinates: 36.410784, -83.763027

Southwest of Leatherwood Baptist Church on Collins Road; several dozen plants on east-facing slope above road.

Bryan J. Peterson: 2012-26A

March 20, 2012

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Madison Co., **ARKANSAS**.
Coordinates: 36.074210, -93.488709

On gentle northeast-facing slope over Sugar Camp Hollow; dozens of plants in woods along east side of County Road 2681. On the west side of the road is a large, open cattle pasture. Some plants located very close to road right-of-way; more plants visible on slope in woods, but property ownership is unknown.

Bryan J. Peterson: 2012-33A

March 22, 2012

Ada Hayden Herbarium (ISC)
Iowa State University

Dirca palustris L.

Madison Co., **ARKANSAS**.
Coordinates: 36.042529, -93.488241

Small population near Dry Creek; approximately twenty plants on slope adjacent to County Road 209 just east of intersection with Hwy 21. Several plants with bright white pubesence on bud scales, and many more with dark brown pubesence.

Bryan J. Peterson: 2012-34A

March 22, 2012