SYSTEMATICS OF *DIRCA* (THYMELAEACEAE) BASED ON ITS SEQUENCES AND ISSR POLYMORPHISMS

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ABSTRACT

The genus *Dirca* consists of three disjunct species of shrubs. *Dirca palustris* is found in the eastern United States and adjacent Canada; *D. occidentalis* is limited to six counties near the San Francisco Bay in California; and the recently discovered *D. mexicana* is known from one isolated population in northeastern Mexico. The three species have been described and classified according to morphological characters, but the morphological evidence does not provide a clear assessment of the relationships among the species. Morphologically, *D. mexicana* most closely resembles *D. occidentalis*, but known biogeographical trends raise doubt regarding how the three species are interrelated. We used molecular techniques to examine and clarify phylogenetic relationships among the three species of *Dirca*. Evidence from Internal Transcribed Spacer (ITS) sequences and Inter-Simple Sequence Repeats (ISSR) polymorphisms confirms the species-level divergence of *Dirca mexicana* and reveals that, despite their morphological similarity, *D. mexicana* and *D. occidentalis* are the most divergent of the three species genetically, while *D. mexicana* and *D. palustris* are the most closely related. The molecular evidence also demonstrates that *Dirca occidentalis* was the first of the three species to diverge and has undergone the greatest degree of differentiation since divergence.

RESUMEN

El género *Dirca* consta de tres especies de arbustos separadas geográficamente. *Dirca palustris* se encuentra en la parte Este de los Estados Unidos y la parte contigua de Canadá; *D. occidentalis* se limita a seis condados alrededor de la bahía de San Francisco en California, EE.UU.; y la recién descubierta *D. mexicana* se conoce solamente de una población aislada en la parte noreste de México. Las tres especies han sido descritas y clasificadas según sus características morfológicas, pero las pruebas morfológicas han sido consideradas como no concluyentes para hacer una evaluación clara de las relaciones entre las especies. Morfológicamente, *D. mexicana* se parece más a *D. occidentalis*, pero las tendencias biogeográficas establecidas crean dudas sobre el grado de parentesco entre las dos especies. Hemos usado técnicas moleculares para examinar y clarificar las relaciones filogenéticas entre las tres especies de *Dirca*. Por las pruebas que encontramos en las secuencias del Espaciador Transcrito Interno (ETI) [Internal Transcribed Spacer (ITS)] y polimorfismos de Repeticiones de Secuencias Intra-Simple (RSIS) [Inter-Simple Sequence Repeats (ISSR)], llegamos a las siguientes conclusiones: 1. *Dirca mexicana* merece ser catalogada como especie; 2. *Dirca occidentalis* fue la primera de las especies en evolucionar, y ha experimentado el mayor grado de diferenciación desde su divergencia; y 3. Contrario a sus morfologías, *D. mexicana* tiene un grado de parentesco más alto, en términos genéticos, con *D. palustris* que con *D. occidentalis*.

INTRODUCTION

The genus *Dirca* L. is comprised of three species of slow-growing, understory shrubs found almost exclusively in nature on steep, west- or north-facing slopes

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above a waterway (Johnson 1994; Nesom & Mayfield 1995; Graves 2004). While *D. palustris* L. is the most common of the species and is found in sparsely distributed colonies over most of eastern North America, the other two species, *D. occidentalis* Gray and *D. mexicana* Nesom & Mayfield, are rare and endemic. *Dirca occidentalis* is isolated to a six-county region surrounding the San Francisco Bay in California, and *D. mexicana* is endemic to only one valley in the Sierra Madre Oriental Mountains of Tamaulipas, Mexico (Nesom & Mayfield 1995; Graves 2004). Although there is interest in the genus due to the obscurity of plants in the wild, the vulnerability of plants in California (Johnson 1994), the discovery of the new species in Mexico (Nesom & Mayfield 1995), and the potential to utilize plants of this genus as shade-tolerant shrubs for managed landscapes (Dirr 1998), no research has been done to determine the phylogenetic relationships among the three species of *Dirca*.

In the most recent treatment of the genus *Dirca*, Nesom and Mayfield (1995) showed the three disjuncts to be morphologically distinct and found that *D. mexicana* more closely resembled *D. occidentalis* than it did *D. palustris*. But, they also questioned the relative similarity of *D. mexicana* and *D. occidentalis* "in view of the well-known pattern of close relationship and disjunction between species of the eastern and southeastern United States and the sierra of northeastern Mexico" (Nesom & Mayfield 1995). Considering biogeographical trends, Nesom and Mayfield (1995) believed the best explanation for the origin of three disjunct species was, first, the isolation of the ancestors of *D. occidentalis* from those of *D. palustris*, and, more recently, the isolation of the ancestors of *D. mexicana* from those of *D. palustris*. Our goals were to resolve the apparent inconsistency between morphological and biogeographical evidence by examining the genotypic relationships among the three species of *Dirca*, to reconstruct the phylogeny of *Dirca* spp. by utilizing methods of molecular systematics, and to determine if molecular evidence supports the classification of *D. mexicana* as a distinct species.

We used two classes of molecular markers, Internal Transcribed Spacer (ITS) sequences and Inter-Simple Sequence Repeats (ISSR), to quantify the genotypic variability of *Dirca*. ITS techniques compare the internal transcribed spacer sequences of the 18S-5.8S-26S nuclear ribosomal DNA. They hold many advantages over other methods, including biparental inheritance, intergenomic variability suitable for phylogenetic inference at the specific, generic, and family levels (Baldwin 1992; Baldwin et al. 1995), and easy amplification with universal primers (White et al. 1990). ITS sequence data are abundant and easily accessible in public databases, enabling direct comparisons among taxa and thus are used extensively for botanical phylogenetics at generic and infrageneric levels (Álvarez & Wendel 2003).

ISSR techniques (Zietkiewicz et al. 1994) are chosen most often for their

capacity to resolve molecular differences below the specific level, but ISSRs are also valued because they sample a large portion of the genome and therefore avoid the bias accompanying phylogenies based on the sequence of only one or a few genes (Schrader & Graves 2004). Used together, these two methods can provide excellent resolution of genetic variability at and below the family level and proved effective for assessing infrageneric differences within the genus *Dirca*.

MATERIALS AND METHODS

Samples of genomic DNA were extracted from leaf tissue of 24 seedlings by utilizing the template preparation service of the DNA Sequencing and Synthesis Facility at Iowa State University. The seeds had been collected from plants of all *Dirca* spp. in their native habitats (Table 1). Eight samples of *D. palustris* and seven samples each of *D. mexicana* and *D. occidentalis* were used to compare genetic variation in *Dirca*. In addition, two samples of *Daphne mezereum* L. (Thymelaeaceae) and one sequence from *Hibiscus rosa-sinensis* L., also from within the Order Malvales, were used as outgroup representatives for phylogenetic analyses (Table 1). *Daphne mezereum* and *H. rosa-sinensis* (GenBank sample, Shi & Yuan 2001) were chosen to establish ancestral-character polarity at the generic and family levels, respectively.

ITS methods.—We amplified the entire ITS region (ITS 1 + 5.8S + ITS 2) of each sample by using the universal primers ITS4 and ITS5 (White et al. 1990), separated the ITS bands by use of agarose-gel electrophoresis, cut out bands, and eluted the purified samples from the agarose with the GenElute™ Gel Extraction Kit (SIGMA, St. Louis, Mo.). For ITS amplification, we used 25-μL reaction mixes that contained 50 ng of template DNA, 0.8 μM of each primer, 600 μM dNTP mix (SIGMA), 1× reaction buffer that contained $Mg(OAc)_2$, and 1.5 units of KlenTaq LA DNA polymerase (SIGMA). Thermocycler conditions were 94° C for 5 min (initial denaturing), 94° C for 1 min (denaturing), 45° C for 1 min (annealing), and 72° C for 2 min (extension), for 35 cycles with the final extension at 72° C for 5 min. The purified samples were sequenced on an Applied Biosystems (ABI) 3100 Genetic Analyzer by using the forward primer (ITS5) and the long-read service of the DNA Sequencing and Synthesis Facility at Iowa State University. We used CLUSTAL X Multiple Sequence Alignment Program (version 1.8) to align sequences for phylogenetic analyses and to confirm the presence of the plant-conserved, 5.8S rDNA motif (Jobes & Thien 1997) in all sample sequences.

ISSR methods.—ISSR fragments for each of the 24 DNA samples were amplified for three replications with each of eight fluorescent 3′-anchored ISSR primers [$(CA)_6RG$, $(AC)_8G$, $(AG)_8YT$, $(CT)_8TG$, $(GTG)_3GC$, $(CA)_6RT$, $(CAC)_3RC$, and $(CTC)_3SG$], which were synthesized at the DNA Sequencing and Synthesis

TABLE 1. Origins of the 25 individuals sampled for ITS and ISSR analysis. All plants sampled are from the Schrader and Graves *Dirca* collection at Iowa State University except *Hibiscus rosa-sinensis*, which was obtained through a BLAST search (Shi & Yuan 2001). Latitude and longitude are according to Global Positioning System (GPS) and are included when known.

Species	Plant #	Accession[z]	Origin	Latitude	Longitude	Analysis
Dirca mexicana	D.mex 1	DMTA02	Tamaulipas	23°59'161" N	99°28'635" W	ISSR
	D.mex 2	DMTA02	Tamaulipas	23°59'161" N	99°28'635" W	ITS/ISSR
	D.mex 3	DMTA02	Tamaulipas	23°59'161" N	99°28'635" W	ITS/ISSR
All in Mexico	D.mex 4	DMTA02	Tamaulipas	23°59'161" N	99°28'635" W	ITS/ISSR
	D.mex 5	DMTA02	Tamaulipas	23°59'161" N	99°28'635" W	ITS/ISSR
	D.mex 6	DMTA02	Tamaulipas	23°59'161" N	99°28'635" W	ITS/ISSR
	D.mex 7	DMTA02	Tamaulipas	23°59'161" N	99°28'635" W	ITS/ISSR
Dirca occidentalis	D.occ 9	DOFT02	Contra Costa Co.	37°49'555" N	122°10'775" W	ITS/ISSR
	D.occ 11	DOAV02	Contra Costa Co.	37°56'015" N	122°18'030" W	ITS/ISSR
	D.occ 12	DOAV02	Contra Costa Co.	37°56'015" N	122°18'030" W	ITS/ISSR
All in California	D.occ 13	DOAV02	Contra Costa Co.	37°56'015" N	122°18'030" W	ITS/ISSR
	D.occ 14	DOAV02	Contra Costa Co.	37°56'015" N	122°18'030" W	ITS/ISSR
	D.occ 15	DOAV02	Contra Costa Co.	37°56'015" N	122°18'030" W	ISSR
	D.occ 16	DOAV02	Contra Costa Co.	37°56'015" N	122°18'030" W	ITS/ISSR
Dirca palustris	D.pal 17	DPLSP01	Boone Co.	41°59'586" N	93°53'058" W	ISSR
	D.pal 18	DPLSP01	Boone Co.	41°59'586" N	93°53'058" W	ITS/ISSR
	D.pal 19	DPLSP01	Boone Co.	41°59'586" N	93°53'058" W	ITS/ISSR
All in Iowa	D.pal 20	DPLSP01	Boone Co.	41°59'586" N	93°53'058" W	ITS/ISSR
	D.pal 21	DPIA01	Boone Co.	41°56'316" N	93°51'595" W	ITS/ISSR
	D.pal 22	DPIA01	Boone Co.	41°56'316" N	93°51'595" W	ITS/ISSR
	D.pal 23	DPRMF01	Clayton Co.	42°48'838" N	91°20'437" W	ITS/ISSR
	D.pal 24	DPRMF01	Clayton Co.	42°48'838" N	91°20'437" W	ISSR
Daphne mezereum	DAPH 25	DMEZ03	Purchased	Forest Farm Nursery	Williams, Oregon	ITS/ISSR
	DAPH 27	DMEZ03	Purchased	Forest Farm Nursery	Williams, Oregon	ITS/ISSR
Hibiscus rosa-sinensis	Blast search	AF460187		NCBI website[y]		ITS

[z] Voucher specimens: *Dirca mexicana*, Accession DMTA02, Tamaulipas, Mexico, *Schrader 124* (ISC). *Dirca occidentalis*, Accession DOFT02, French Trail, Contra Costa Co., Calif., *Schrader 125* (ISC). *Dirca occidentalis*, Accession DOAV02, Aqua Vista, Contra Costa Co., Calif., *Schrader 126* (ISC). *Dirca palustris*, Accession DPLSP01, Ledges State Park, Boone Co., Iowa, *Schrader 127* (ISC). *Dirca palustris*, Accession DPIA01, Iowa Arboretum, Boone Co., Iowa, *Schrader 128* (ISC). *Dirca palustris*, Accession DPRMF01, Retz Memorial Forest, Clayton Co., Iowa, *Schrader 129* (ISC). *Daphne mezereum*, Accession DMEZ03, Purchased from Forest Farm Nursery, Williams, Oregon, *Schrader 130* (ISC).

[y] National Center for Biotechnology Information (NCBI). www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=Nucleotide&list_uids=32364883&dopt=GenBank

Facility at Iowa State University. Optimization reactions were run to determine proper reaction conditions and reagent concentrations for consistent PCR amplification. Thermocycler conditions for ISSR-PCR were 94° C for 5 min (initial denaturing), 94° C for 30 s (denaturing), primer-specific temperatures (see below) for 45 s (annealing), and 72° C for 2 min (extension), for 30–33 cycles with the final extension at 72° C for 5 min. Annealing temperatures for the eight primers were 47° C for $(CA)_6RG$, 52° C for $(AC)_8G$, 56° C for $(AG)_8YT$, 52° C for $(CT)_8TG$, 56° C for $(GTG)_3GC$, 48° C for $(CA)_6RT$, 52° C for $(CAC)_3RC$, and 52° C for $(CTC)_3SG$. In our 25-μL reaction mixes, we used 50 ng of template DNA, 1.2 μM of primer, 300 μM dNTP mix (SIGMA), 1× reaction buffer that contained $Mg(OAc)_2$, and 1 unit of KlenTaq LA DNA polymerase (SIGMA).

Amplification products were processed at the DNA Sequencing and Synthesis Facility at Iowa State University. Applied Biosystems (ABI) 377 automated DNA sequencing systems separated the DNA by electrophoresis and collected the gel image. Image data were analyzed by using ABI PRISM™ GeneScan® software that resolves DNA fragment length differences as small as one base pair. ISSR bands (loci) were scored as "1" for band presence and "0" for band absence. Only bands that appeared in at least two of the three replications were considered present. A locus was any fragment length that was present in at least one sample. The resulting two-state (1 · 0) data matrices for the eight primers were combined to form a cumulative data set for assessing molecular relationships among the three species of *Dirca*. Data from three of the primers, $(CA)_6RG$, $(AC)_8G$, and $(AG)_8YT$, were compared with the results of Schrader and Graves (2004) to help assess the relative taxonomic distances expected for specific and subspecific hierarchical levels according to ISSR methods (Tables 2 and 3).

Data analysis.—Cladistic analyses were performed by using PHYLIP (Phylogeny Inference Package; Felsenstein 1995). We used the Dnapars program for Wagner parsimony (Kluge & Farris 1969) analysis of ITS data and the Mix program for Wagner parsimony analysis of ISSR data. The Seqboot program was used for bootstrap (Felsenstein 1985) and jacknife (Farris et al. 1996) analyses (1000 resamplings each), and the Neighbor program for neighbor-joining analyses (Felsenstein 1995). Genetic distances for ITS analyses were generated under the Kimura 2-parameter model (Kimura 1980) by using the Dnadist program of PHYLIP, and genetic distances for ISSR analyses were Euclidean distances (Sneath & Sokal 1973). We compared and contrasted our ITS and ISSR phylogenies, a procedure termed "cross matrix disparity" by Bateman (1999), then merged the two data sets for a "simultaneous analysis" (Nixon & Carpenter 1996) using unweighted distances (Sneath & Sokal 1973) from the two data sets.

RESULTS

ITS.—Sequencing of the ITS region provided complete sequences for ITS 1, the 5.8S rRNA gene, and ITS 2 and provided partial sequences for the 18S (32 nucle-

otides) and 26S (22 nucleotides) rRNA genes. The ITS region varied in length among the four species we evaluated (630 bp for *D. mexicana*, 617 bp for *D. occidentalis*, 625 bp for *D. palustris*, and 596 bp for *D. mezereum*) and contained ample sequence variation for species-level phylogenetic analysis. The 5.8S rRNA gene was 165 base pairs long in all samples and the sequence was identical in *D. mexicana* and *D. palustris*, with only one site difference for *D. occidentalis* and four site differences for *D. mezereum*, one of which was common to *D. occidentalis*. There was no intraspecific sequence variation among ITS samples from *D. occidentalis*, *D. palustris*, nor *D. mezereum*. There was variation at six sites among the six ITS samples of *D. mexicana* (five insertion/deletions and one transversion, all in ITS 1), but the consensus sequence was identical to the sequence of one of those samples (D.mex 2).

In our phylogenetic analysis, exhaustive searches produced single most-parsimonious trees with each of the two chosen outgroups, *D. mezereum* and *H. rosa-sinensis*, showing 196 and 496 evolutionary steps, respectively. The trees agreed in topology and revealed that, within the genus *Dirca*, the ancestral line of *D. occidentalis* was the first to diverge (Fig. 1 and 2). Bootstrap and jacknife percentages (100% for all clades) showed very strong support for this topology, and our results using the family-level root (outgroup *H. rosa-sinensis*) support the choice of *D. mezereum* as a suitable outgroup for phylogenetic reconstruction of *Dirca*.

Sequence divergence (Kimura 2-parameter distance) between *D. occidentalis* and the other two *Dirca* species (*occidentalis* to *mexicana* = 0.0592, *occidentalis* to *palustris* = 0.0560) was much greater than the divergence between *D. mexicana* and *D. palustris* (0.0074). Results of the neighbor-joining analyses reveal both a much earlier divergence of *D. occidentalis* than the divergence of the other two species and greater differentiation than the other two species since their times of divergence (Figs. 1 and 2). This feature is particularly apparent when the phylogeny is constructed by using a generic-level root (Fig. 1), but is still evident when using a family-level outgroup (Fig. 2).

ISSR.—Amplification with the eight fluorescent 3′-anchored primers yielded 709 ISSR loci (fragment lengths) across the four species. The fine resolution of ISSR techniques is illustrated by the high degree of polymorphisms found among the three species of *Dirca* and the high number of species-specific loci (Table 2). Comparing these results with the results of an earlier, subspecific study that involved the same methods and three of the same ISSR primers (Schrader and Graves 2004), confirmed species-level divergence among the three taxa of *Dirca*. For the three primers used in both studies, the three species of *Dirca* had nearly double the percentage of polymorphic loci and taxon-specific loci of those seen among the three subspecies of *Alnus maritima* (Marsh.) Muhl. ex Nutt. (Table 2). The differentiation revealed in the number of taxon-specific loci found among the three species of *Dirca* was consistent with the

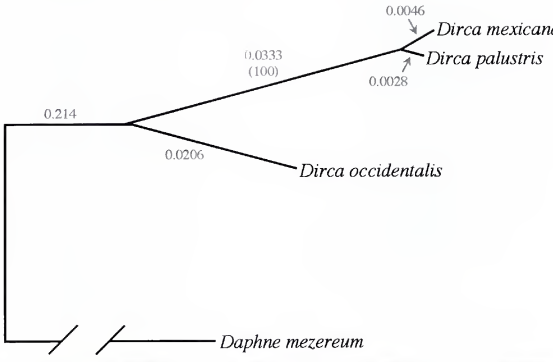

FIG. 1. ITS neighbor-joining dendrogram showing the inferred phylogenetic relationship among the three species of *Dirca*. Topology indicates that of the single most-parsimonious tree. Numbers indicate relative branch lengths; numbers in parentheses are both the bootstrap and jacknife percentages, which were identical. *Dahne mezereum*, another member of Thymelaeaceae, was used as the outgroup in order to establish ancestral-character polarity of the ITS sequence.

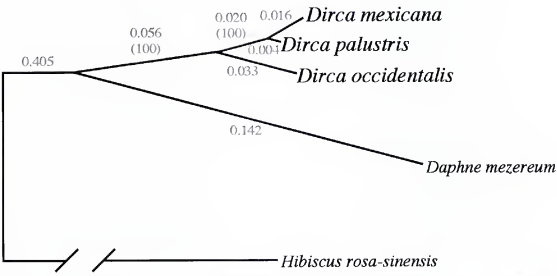

FIG. 2. ITS neighbor-joining dendrogram showing the inferred phylogenetic relationship among the three species of *Dirca* and another member of Thymelaeaceae, *Dahne mezereum*. Topology indicates that of the single most-parsimonious tree. Numbers indicate relative branch lengths; numbers in parentheses are both the bootstrap and jacknife percentages, which were identical. *Hibiscus rosa-sinensis*, another member of Order Malvales, was used as the outgroup to establish ancestral-character polarity of the ITS sequence.

TABLE 2. Percentage of polymorphic loci and percentage and number of taxon-specific bands resolved by using three (*Dirca* and *Alnus*) and eight (*Dirca*), 3'-anchored ISSR primers. Results for *Dirca* are from the present study; results for *Alnus* were obtained by Schrader and Graves (2004) by using the same methods and three of the same primers as the present study.

	Primers				
	$(CA)_6RG$	$(AG)_8YT$	$(AC)_8G$	Three primers	All eight primers
% of polymorphic loci					
Dirca species	87	95	88	90	83
Alnus maritima subspecies	41	55	46	48	
% Taxon-specific loci					
Dirca species	52	73	47	57	55
Alnus maritima subspecies	15	31	14	22	
# Taxon-specific loci					
Dirca mexicana	7	17	13	37	112
Dirca palustris	9	10	6	25	67
Dirca occidentalis	16	16	11	43	164
Alnus maritima					
subsp. *oklahomensis*	1	3	0	4	
subsp. *georgiensis*	0	1	2	3	
subsp. *maritima*	2	4	0	6	
Alnus japonica	9	16	12	37	

species-level differentiation in taxon-specific loci of the outgroup *Alnus japonica* (Thunb.) Steud. for each and all primers and was over four times that shown among the subspecies of *A. maritima*. Further evidence of species-level divergence was revealed by analysis of the genetic distances between the taxa (Table 3). The distance between the least divergent pair, *D. mexicana* and *D. palustris*, (103) is over five times greater than that of the most divergent subspecies of *A. maritima* (19).

After an exhaustive search, phylogenetic analysis of ISSR data produced a single most-parsimonious tree of 813 evolutionary steps (Fig. 3), and bootstrap and jacknife percentages (100% for both) showed very strong support for this topology. Consistent with the ITS results, ISSR data verified that *D. occidentalis* was the first of the three *Dirca* species to diverge (Fig. 3). Euclidean distances between species based on data from all eight primers were 410 for *D. occidentalis* and *D. palustris*, 392 for *D. occidentalis* and *D. mexicana*, and 302 for *D. palustris* and *D. mexicana*. These genetic distances and branch lengths derived from ISSR markers showed greater relative distance between *D. mexicana* and *D. palustris* than obtained from ITS, indicating a more uniform level of divergence among the three species (Fig. 3). Although divergence was shown to be more uniform,

TABLE 3. Comparative Euclidean distances for species (*Dirca*) and subspecies (*Alnus maritima*) obtained by the same methods and same three ISSR primers. Results for *Alnus maritima* were derived from Schrader and Graves (2004).

	Euclidean distances
Species level (*Dirca*)	
Dirca mexicana – *Dirca occidentalis*	110
Dirca mexicana – *Dirca palustris*	103
Dirca occidentalis – *Dirca palustris*	119
Subspecies level (*Alnus maritima*)	
oklahomensis – *georgiensis*	15
oklahomensis – *maritima*	19
georgiensis – *maritima*	12

ISSR results confirmed that *D. mexicana* and *D. palustris* are the most closely related of the three species. Our unweighted, simultaneous analysis of ITS and ISSR data (Fig. 4) shows the best synthesis for the phylogeny of *Dirca* based on all available molecular evidence.

DISCUSSION

Based on the results of ITS alone, we might conclude that *Dirca mexicana* and *D. palustris* could best be considered as the same species, a conclusion that would contradict the morphological evidence of Nesom and Mayfield (1995). With ITS, the mean genetic distance from *D. occidentalis* to these two species is nearly eight times greater than the distance between *D. mexicana* and *D. palustris*, and the neighbor-joining phylogeny, produced when using generic-level ancestral character as the root, illustrates how closely related the ITS regions of *D. mexicana* and *D. palustris* are to each other (Fig. 1). Results of ISSR analysis, however, provide conclusive evidence that *D. mexicana* and *D. palustris* have diverged sufficiently to be considered separate species, and they indicate that divergence of the three species is more uniform than indicated by ITS analysis (Table 2 and 3, Fig. 3). Although a suitable explanation for the seemingly contradictory levels of divergence indicated by ITS and ISSRs could be that different genetic markers may be differentially affected by occurrences such as interspecific gene flow or reticulate evolution (Comes & Abbott 1999), a more obvious explanation can be found in a closer examination of the two ITS phylogenies. Including family-level ancestral character (outgroup *H. rosa-sinensis*) in the analysis led to two important insights. First, there is a high degree of differentiation (long branch length) of *D. occidentalis* and *D. mezereum* that is evident after their divergence (Fig. 2), and this differentiation skews the tree produced by using *D. mezereum* as the outgroup (Fig. 1) causing *D. mexicana*

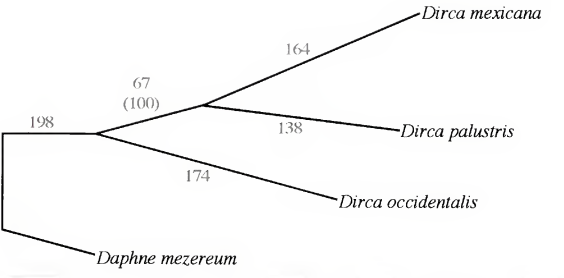

FIG 3. ISSR neighbor-joining dendrogram showing the inferred phylogenetic relationship among the three species of *Dirca*. Topology indicates that of the single most-parsimonious tree. Numbers indicate relative branch lengths; numbers in parentheses are both the bootstrap and jacknife percentages, which were identical. *Dahne mezereum*, another member of Thymelaeaceae, was used as the outgroup to establish ancestral-character polarity of ISSR banding patterns.

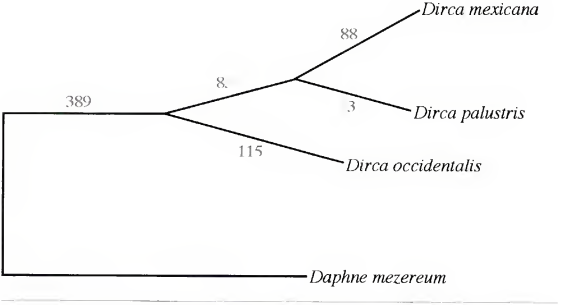

FIG. 4. Simultaneous ITS and ISSR neighbor-joining dendrogram using unweighted Kimura and Euclidean distances, respectively. *Dahne mezereum*, another member of Thymelaeaceae, was used as the outgroup to establish ancestral-character polarity.

and *D. palustris* to be placed further from *D. occidentalis* than they would be otherwise. Secondly, the phylogeny produced with family-level ancestral character in the root corrects for this disproportional differentiation and shows a more uniform species-level divergence between the three *Dirca* species, making the phylogeny more consistent with the results of the ISSRs. Together, the ISSR and ITS analyses support the species designation for *D. mexicana* established by Nesom and Mayfield (1995). These results also reinforce the principle that more than one genetic marker should be used in molecular systematics investigations (Hollingsworth et al. 1999) and that analysis of cross-matrix disparity can be a valuable method of clarifying phylogenetic features (Bateman 1999).

Contrary to the morphological evidence, both ITS and ISSR analyses indicate that *Dirca mexicana* is more closely related, genetically, to *D. palustris* than it is to *D. occidentalis*. This finding, and the findings that *D. occidentalis* was both the first of its genus to diverge and the most differentiated of the three species (Figs. 1–4), help to explain the apparent inconsistency between the morphological and biogeographical evidence noted by Nesom and Mayfield (1995). Although there is a greater morphological similarity between *D. mexicana* and *D. occidentalis*, they are the most divergent of the three species genetically (Figs. 1–4). These findings are consistent with the hypothesis of Nesom and Mayfield (1995) based on biogeography, that *Dirca* was probably continuous across North America as early as the Eocene, and disjunction between *D. occidentalis* and *D. palustris* may have taken place before or around the same time as the movement of *Dirca* into Mexico. This hypothesis seems especially well supported by our ITS and simultaneous phylogenies (Figs. 1, 2, and 4).

The typical ecological niche for the genus *Dirca* is considered to be the cool moist slopes of mature temperate deciduous forests. Both *D. palustris* and *D. mexicana* are found almost exclusively in such settings, and even though it has adapted to a different environment, *D. occidentalis* is struggling to survive amidst a drying climate, removal of overstory trees, and competition from evergreen trees and shrubs (Johnson 1994; Graves 2004). As mentioned by Nesom and Mayfield (1995), paleobotanical evidence suggests that *Dirca* was continuous across the midlatitudes of North America as part of the Eocene/Oligocene expansion of temperate deciduous vegetation that took place after the partial regression of the Cretaceous epeiric sea (Graham 1993). In fact, members of Thymelaeaceae are documented in the late Eocene flora of central Colorado (MacGinitie 1953; Graham 1993). In geologic time, this extensive range was probably short lived. Cooling during the middle Miocene brought the encroachment of coniferous forests from the north (Leopold & Denton 1987), and by the middle to late Miocene, colder winter temperatures and reduced summer rainfall in the area of the Great Plains had initiated the development of prairie grasslands (Graham 1993). While climatic cooling brought the isolation of eastern and western deciduous forests and, most likely, the eastern and western representa-

tives of *Dirca*, it also enabled the movement of eastern deciduous forest element into eastern Mexico following rapid southward retreat of the lingering Mississippi Embayment by the middle to late Miocene (Graham 1973, 1993). Finally, the precursors to modern *D. palustris* and *D. mexicana* probably became disjunct as prairie and coniferous communities in western and southwestern North America spread during the Pliocene and elements of the broad-leaved deciduous forest that had extended into eastern Mexico became isolated (Graham 1973, 1993).

One phenotypic manifestation of the high level of differentiation seen in *D. occidentalis* is its obligate summer dormancy. Only *D. occidentalis* undergoes this drought-deciduous summer dormancy, which coincides with the dry season in the San Francisco Bay area. Trials we have conducted in a greenhouse indicate that this annual phenological event cannot be overcome by manipulation of the environment. The absence of this drought-deciduous trait in the other two species of *Dirca*, and, to the best of our knowledge, the rest of Thymelaeaceae, suggests that considerable change has taken place in *D. occidentalis* since its divergence to ensure survival in its Mediterranean climate (Freitas 1997). Such adaptation is the most plausible explanation for the continued existence of this disjunct species of *Dirca*. Without a means of drought avoidance, it is likely that the precursors of *D. occidentalis* would have expired along with the rest of the western *Dirca* element as western climates became increasingly dry. It is believed that during the Pliocene, the Cascade-Sierra Nevada and the Coast Ranges reached sufficient heights to create an effective rain shadow over the Basin and Range Province, resulting in a change from mesic and summer-wet to the xeric and summer-dry conditions that exist today (Axelrod 1986; Graham 1993). This trend most likely forced *D. occidentalis* into its meager coastal distribution, while promoting selection of the summer-deciduous habit.

The lower level of differentiation in *Dirca palustris* revealed by all four phylogenetic dendrograms should be considered consistent with its much larger potential gene pool and, until recently, its fairly uniform habitat. Because the fundamental niche of *D. palustris* is mature-forest understory, it is likely that, except for the temporary intrusion of glaciers and their adjacent boreal forest biome (Delcourt & Delcourt 1993), the genetic aggregate of *D. palustris* was continuous across eastern North America until the harvest of old-growth forests within the last 300 to 400 years. Future examination of the genetic variation within *D. palustris* should be performed to test this hypothesis and to provide an even clearer picture of the systematics of genus *Dirca*.

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