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AT THE INTERFACE OF PHYLOGENETICS AND POPULATION GENETICS, THE PHYLOGEOGRAPHY OF *DIRCA OCCIDENTALIS* (THYMELAEACEAE)[1]

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Dirca occidentalis is a rare shrub indigenous to only six counties near the San Francisco Bay in California, United States. We used intersimple sequence repeat (ISSR) markers and automated genotyping to probe 29 colonies of *D. occidentalis* from four geographically disjunct populations (East Bay, North Bay, Salmon Creek, and Peninsula) and used methods of phylogenetics and population genetics to model variation across the species. Results show that the four disjunct populations are genetically isolated and have undergone divergence. Phylogenetic analyses indicate that the East Bay population was the first to diverge, followed by the North Bay, then the Salmon Creek and Peninsula populations. This order of divergence suggests an intriguing natural history for *D. occidentalis* that is explained by the dynamic geological and climatic history of the Bay Area. Spatial genetic structure detected for the species suggests an interaction of four factors: limited seed dispersal, clonal regeneration, distances traveled by pollinators, and genetic isolation of the four populations. Genetic diversity within the North Bay and Salmon Creek populations is low, indicating poor ecological fitness and risk of decline. ISSRs resolved phylogeographic structure within *D. occidentalis*, results unattainable with ITS methods, and the integration of tools of phylogenetics and population biology led to an enhanced understanding of this endemic species.

Key words: *Dirca occidentalis*; endemic; genetic structure; intersimple sequence repeats (ISSRs); intraspecific; natural history; phytogeography; plant ecology; spatial autocorrelation; Thymelaeaceae.

Studies pertaining to principles and processes that govern geographical distributions of genealogical lineages within species have enhanced our understanding of evolution, population genetics, and biogeography over the past few decades (Avise et al., 1987; Avise, 2000). Impacts of this phylogeographic research in plant systems have lagged behind those of animal systems due primarily to difficulty with identifying and utilizing genetic markers that resolve appropriate levels of intraspecific variation (Schaal et al., 1998). While phylogeographic studies of animals rely heavily on data gleaned from the mitochondrial genome (mtDNA), in plants these and most other genetic markers that rely on sequence information have inadequate variation for consistent use at the intraspecific level (Schaal et al., 1998; Pelser et al. 2003). Difficulty in applying sequence data to intraspecific genetic analyses in plants has helped fuel the development and increased use of methods such as intersimple sequence repeats (ISSRs), random amplified polymorphic DNA (RAPDs), and amplified fragment length polymorphisms (AFLPs) that use polymerase chain reactions (PCR) to fingerprint variations in hypervariable DNA (Bussell et al., 2005; Archibald et al., 2006b). Such markers have been applied extensively at the intraspecific level (Fang et al., 1998; Moreno et al., 1998; Raina, 2001; Wolfe and Randle, 2001; Iruela et al., 2002; Potter et al., 2002; Schrader and Graves, 2004a) and are deemed appropriate for phylogenetic analyses below taxonomic levels at which ITS (internal transcribed spacer) and other variable sequences can supply sufficient information, a level of resolution at the interface of phylogenetics and population genetics (Bussell et al., 2005; Archibald et al., 2006a).

Endemic species of plants with disjunct distributions are particularly interesting subjects for the study of phylogeography (Conte and Cristofolini, 2000; Thompson, 2005). Despite a wealth of knowledge amassed for many of these taxa, factors that account for their peculiar distributions are often elusive (Gankin and Major, 1964; Simurda and Knox, 2000), and answers are often found outside the general models of ecology, biogeography, and population genetics (Broadhurst et al., 1999; Mota et al., 2002; Mengoni et al., 2003; Ellison and Farnsworth, 2005). *Dirca occidentalis* Gray, a deciduous shrub found only near the San Francisco Bay, is an intriguing model species in which to explore the nature of disjunct endemics. It is the only member of the Thymelaeaceae indigenous to California, and its disjunct populations occur within an area of only 8000 km^2 that is rich in biogeographical history and variation. While the species typically occurs within small niches characterized by sloped topography, shade, and fog (Johnson, 1994; Graves, 2005), the range of habitats in which *D. occidentalis* occurs is remarkable. For example, at Jasper Ridge Biological Preserve in San Mateo County, *D. occidentalis* is most prevalent in open and riparian deciduous woodlands, where clusters of more than 50 plants exist, but the species also occupies chaparral communities (Ackerly et al., 2002), open scrublands, and broadleaf evergreen forests. It is paradoxical that *D. occidentalis* persists within such diverse vegetative associations and yet is limited to such a small geographical area. Observations over the last few decades indicate that populations of *D. occidentalis* are in decline (Johnson, 1994). Slow growth, low fecundity, limited seed dispersal, and poor seed germination may be restricting the capacity of *D. occidentalis* to compete within its native range (Johnson, 1994; Schrader and Graves, 2005). Low frequencies of sexual reproduction, and the recently confirmed

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capacity of the species to reproduce asexually from rhizomes, may restrict genetic diversity within populations (Graves, 2004).

Distributed northwest, south, and east of the San Francisco Bay, *D. occidentalis* occurs in four discrete areas (East Bay, North Bay, Salmon Creek, and Peninsula; Fig. 1) separated by distances of at least 29 km. The species is relatively abundant in portions of San Mateo and Santa Clara counties on the Peninsula south of San Francisco (Fig. 1; within red line) and in Contra Costa and Alameda counties in the East Bay (Fig. 1; within violet line). *Dirca occidentalis* is less prevalent in the North Bay (Fig. 1; within blue line) where Howell (1970) described only four locations in Marin County where the species occurs. Best et al. (1990) described the occurrence of *D. occidentalis* near Salmon Creek (Fig. 1; within yellow line) in Sonoma County, where we observed only about 50 mature plants within approximately 50 hectares.

Wells and Richmond (1995, p. 461) define the term population as "a group of conspecific individuals that is demographically, genetically, or spatially disjunct from other groups of individuals." By this definition, the four groups of *D. occidentalis* should be considered distinct populations on the basis of their spatial disjunction. We hypothesized that the four geographically defined populations are genetically isolated and that genetic divergence has taken place since their isolation. Our objective was to test this hypothesis, to characterize the genetic structure and phylogeography of *D. occidentalis*, and to advance our understanding of the geographical and natural history of this endemic shrub. While no obvious phenotypic differences have yet been noted among plants of the four populations, and research using ITS sequence data failed to detect variation within *D. occidentalis* (Schrader and Graves, 2004b), intraspecific variation can be resolved by using hypervariable, PCR-based markers (Zietkiewicz et al., 1994; Schrader and Graves, 2004a, b). We used ISSRs with fluorescent primers and digital imaging to quantify variation within and among the four known populations of *D. occidentalis*. A large portion of the eukaryotic genome is screened using ISSR markers (Wolfe and Liston, 1998; Abbot, 2001), which are considered more effective, more reliable, and more reproducible than other hypervariable DNA markers such as RAPDs (Nagaoka and Ogihara, 1997; Moreno et al., 1998; Wolfe and Liston, 1998). Phylogenetic inferences and derivations of genetic structure within and among plant populations have been based on results obtained using ISSRs (Bussell et al., 2005; Archibald et al., 2006a, b). We used phylogenetic and spatial analyses of ISSR banding patterns to model the genetic structure of *D. occidentalis* and to provide insights regarding the cause and timing of its disjunct distribution. A genetic structure was identified that is strongly linked to geography and plausibly explained by the biology of *D. occidentalis* and its associated ecosystem. Our results were enriched by considering genetic and biological information within the context of the geologic and climatic history of the San Francisco Bay region, illustrating the potential value of integrating phylogenetics and population genetics in studies of rare endemics.

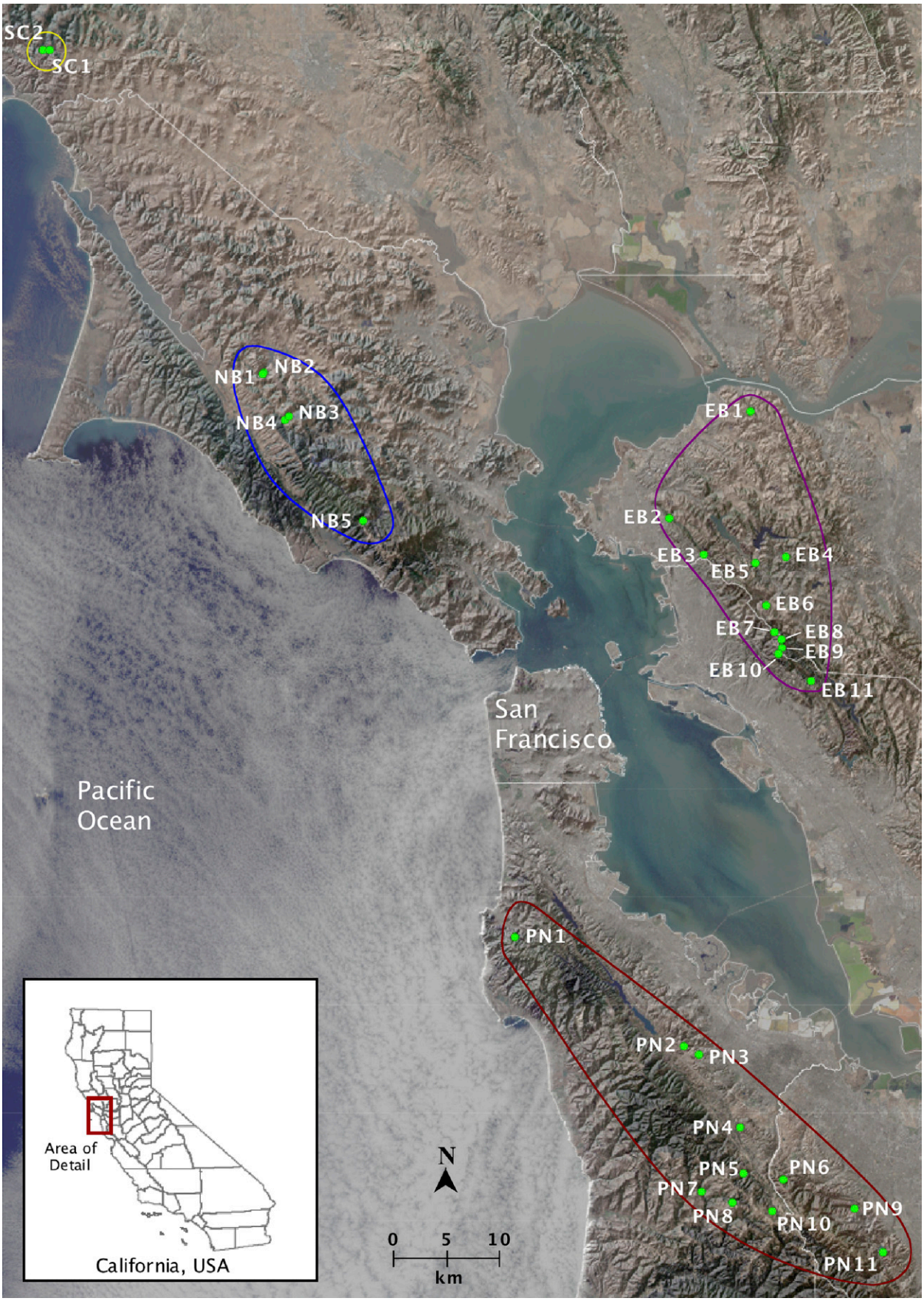
MATERIALS AND METHODS

Field surveys, rare-plant databases, and interviews with local botanists led to identification of numerous subpopulations of *D. occidentalis* throughout its distribution (Fig. 1). Special efforts were made to find subpopulations at geographical extremes within each population. From one to 12 plants were sampled within subpopulations. The number of samples for each subpopulation was based on the estimated subpopulation size, and the number of subpopulations assessed for each population was based on relative population size. This proportional sampling helped facilitate an accurate comparison of genetic diversity and differentiation (Hoel, 1966). We avoided sampling from neighboring plants when possible to minimize the chance of sampling ramets (Graves, 2005). Leaves from each plant were removed with a disinfected razor blade, placed in separate plastic bags with ≈20 g of charged silica-gel desiccant, and dried for ≥3 d. DNA was extracted from leaves of 60, 59, 43, and 16 plants of the East Bay, Peninsula, North Bay, and Salmon Creek populations, respectively, and voucher specimens were deposited in herbaria (Table 1). DNA samples from three plants of *Dirca palustris* L., the species most closely related to *D. occidentalis* (Schrader and Graves, 2004b), were extracted for use as the outgroup for analyses.

Fragments for each DNA sample were amplified for three replications with each of five fluorescent, 3′-anchored ISSR primers [$(CA)_6$RG, $(AC)_8$G, $(GTG)_3$GC, $(CAC)_3$RC, and $(CTC)_3$SG]. Optimization reactions were run to determine reaction conditions and reagent concentrations for consistent PCR amplification. Thermalcycler conditions for ISSR-PCR were 94°C for 5 min (initial denaturing), 30–33 cycles at 94°C for 30 s (denaturing), primer-specific temperatures (detailed next) for 45 s (annealing), and 72°C for 2 min (extension); with the final extension at 72°C for 5 min. Annealing for the five primers was done at 47°C for $(CA)_6$RG, 52°C for $(AC)_8$G, 56°C for $(GTG)_3$GC, 52°C for $(CAC)_3$RC, and 52°C for $(CTC)_3$SG. These primers were chosen because they provided ample polymorphisms but also resulted in ≥20% monomorphism across all genotypes in the study, a practical level of similarity recommended by Bussell et al. (2005). In our 25-μL reaction mixes, we used 50 ng of template DNA, 1.2 μmol of primer, 300 μmol dNTP mix (Sigma, St. Louis, Missouri, USA), 1× reaction buffer that contained $Mg(OAc)_2$, and one unit of KlenTaq LA DNA polymerase (Sigma).

Amplification products were processed using Applied Biosystems (ABI; Foster City, California, USA) 377 automated DNA sequencing systems that separated the DNA by electrophoresis and collected the gel image. Image data were analyzed using ABI PRISM GeneScan software, which resolves differences in DNA fragment length as small as one base pair. Bands (loci) representing ISSR amplification fragments between 100 and 500 base pairs in length were scored as "1" for presence and "0" for absence. Only bands that appeared in at least two of the three replications were considered present. A locus was any fragment length present in at least one sample. The resulting two-state (1 • 0) data matrices for the five primers were combined to form a cumulative data set for assessing molecular relationships. Banding profiles of the 12 unresolved genotypes (see Results), those that had the same banding profile as at least one other sample, were included in all analyses because our sampling strategy avoided neighboring plants, and failure to resolve differences by using five markers is not definitive evidence of clonality (Esselman et al., 1999; Lamote et al., 2005).

Ward's minimum variance cluster analyses were performed using JMP, version 3.2.6 (SAS Institute, Cary, North Carolina, USA). Cladistic and phylogenetic analyses were performed using PHYLIP (Phylogeny Inference Package; Felsenstein, 1995). We used the Mix program for Wagner parsimony analysis of ISSR data. The consistency index (CI; Kluge and Farris, 1969) and retention index (RI; Farris, 1989) were calculated for maximum-parsimony trees. The Seqboot program was used for bootstrap (Felsenstein, 1985) and "delete-half" jackknife (Farris et al., 1996) analyses (1000 resamplings each) and the Neighbor program for neighbor-joining analyses (Felsenstein, 1995). Genetic distances for neighbor-joining analyses were Nei–Li distances (Nei and Li, 1979) and for population-genetic analyses were Euclidean distances (Sneath and Sokal, 1973; Huff et al., 1993). The outgroup of *D. palustris* was used to establish ancestral-character polarity of ISSR banding patterns for cluster, parsimony, and neighbor-joining analyses because *D. palustris* is the species most closely related to *D. occidentalis* (Schrader and Graves, 2004b). Consensus-banding profiles established from the raw banding profiles of individual samples were used to assess relationships among populations and subpopulations so that the genotypic makeup of populations and subpopulations was represented by extensive sampling rather than by one individual from each. In the consensus profiles, a band was deemed present if it occurred in ≥50% of the samples from a population or subpopulation and absent if it occurred in <50% of the samples. Analysis of molecular variance (AMOVA; Excoffier et al., 1992), Mantel tests (Mantel, 1967), and analyses of spatial autocorrelation (Smouse and Peakall, 1999) were performed using the GenAlEx population genetics package (Peakall and Smouse, 2006). AMOVA,


SC2
SC1
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NB3
NB4
NB5
EB1
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EB3
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TABLE 1. Population and subpopulation locations, number of samples, and voucher information for phylogeographic analysis of *Dirca occidentalis* endemic to the San Francisco Bay region of California. *Dirca occidentalis* exists as four populations (East Bay, North Bay, Salmon Creek, and Peninsula) distinguishable both genetically and geographically.

Population and subpopulation	Latitude (N)	Longitude (W)	Elevation (m a.s.l.)	No. samples	Voucher (ISC) (*W.R. Graves*)
East Bay					
EB1	38°02.132′	122°12.864′	187	9	1
EB2	37°56.469′	122°18.344′	116	4	60
EB3	37°54.580′	122°16.066′	180	6	12, 13
EB4	37°54.364′	122°10.589′	211	2	10, 11
EB5	37°54.063′	122°12.643′	87	6	18, 20
EB6	37°51.850′	122°11.932′	274	7	24, 25
EB7	37°50.376′	122°11.373′	383	2	53
EB8	37°50.030′	122°10.916′	356	2	55
EB9	37°49.564′	122°10.889′	358	5	48, 50
EB10	37°49.277′	122°11.132′	423	7	41, 42
EB11	37°47.797′	122°08.978′	199	10	31, 38
North Bay					
NB1	38°04.299′	122°45.472′	49	11	61
NB2	38°04.276′	122°45.563′	26	12	72, 74
NB3	38°01.986′	122°43.793′	96	1	103
NB4	38°01.790′	122°44.093′	239	8	95, 96
NB5	37°56.484′	122°38.846′	203	11	85, 88
Salmon Creek					
SC1	38°21.405′	122°59.846′	34	8	112, 113
SC2	38°21.390′	123°00.302′	53	8	105, 109
Peninsula					
PN1	37°34.373′	122°28.838′	196	8	165, 166
PN2	37°28.536′	122°17.673′	126	5	150, 151
PN3	37°28.106′	122°16.643′	106	4	163
PN4	37°24.250′	122°13.953′	133	9	120
PN5	37°21.752′	122°13.762′	317	5	140
PN6	37°21.457′	122°11.134′	288	6	159
PN7	37°20.823′	122°16.532′	217	4	176
PN8	37°20.237′	122°14.509′	485	4	130
PN9	37°19.887′	122°06.385′	166	5	145
PN10	37°19.760′	122°11.873′	553	2	173
PN11	37°17.564′	122°04.544′	209	7	133

Note: All vouchers cited are deposited at Ada Hayden Herbarium, Iowa State University (ISC).

Mantel, and spatial autocorrelation analyses were calculated for 999 permutations or bootstrap resamplings to test significance.

RESULTS

Amplification of 181 DNA samples from *D. occidentalis* and *D. palustris* (the outgroup) with five 3′-anchored ISSR primers yielded 565 loci. Monomorphism across all samples (including *D. palustris*) was 21% and was 66% among the samples of *D. occidentalis*. AMOVA analysis with 178 individuals from the 29 subpopulations and four populations (Table 1, Fig. 1) revealed that 41% of genetic variation was partitioned among individuals within subpopulations, 22% among subpopulations, and 37% among populations. The East Bay population had the most genetic diversity. It had both the greatest number of ISSR loci and the greatest percentage of polymorphic loci among samples within each population (Table 2). Genotypes of the East Bay population also had the most population-specific loci (Table 2). Analyses comparing the ISSR banding profiles of the 178 plants of *D. occidentalis* sampled revealed the presence of five sets of plants (12 plants total) that were not resolved by our five ISSR markers; each of these genotypes had the same banding profile as at least one other sample in its set. Four of the sets were from the North Bay population (one set of four plants, three sets of two plants), and one set (of two plants) was from the Peninsula population. All five sets contained plants from a single subpopulation, all within 5 m of one another. We estimated by spatial analysis of the remaining samples that 57 plants within 5 m of each other (≈83% of plants within 5 m of another plant) were resolved as separate genotypes by our ISSR markers.

Initial hierarchical cluster analyses of allele profiles of all 178 DNA samples from *D. occidentalis* revealed strong clustering of genotypes within their geographically defined populations (data not shown). Ward's minimum variance method yielded four main clusters with 100, 93, 81, and 69% of samples from the North Bay, East Bay, Peninsula, and Salmon Creek, respectively, grouped within their geographical populations. Samples that did not group within the four main clusters were placed in small clusters, mostly with other samples from their

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Fig. 1. Geographic distribution of *Dirca occidentalis*, a rare shrub endemic to four areas surrounding the San Francisco Bay in California. Lines indicate geographical limits of populations of *D. occidentalis*: blue, North Bay (NB); violet, East Bay (EB); red, Peninsula (PN); yellow, Salmon Creek (SC). Distribution within each subregion is patchy, and plants do not occur consistently throughout the delineated areas. Names and locations of subpopulations sampled in this study are indicated by green dots and adjacent alphanumeric labels.

population. In the hierarchical dendrogram, the East Bay group was located closest to the outgroup cluster (*D. palustris*) and was the first to diverge, followed by the North Bay group, then the Salmon Creek and Peninsula groups.

Combining individual allele profiles into subpopulation consensus profiles reduced the within-subpopulation variation and helped to resolve the phylogenetic structure of *D. occidentalis*. Parsimony analysis of 29 geographically defined subpopulations produced 19 most-parsimonious trees requiring 541 steps (CI = 0.723, RI = 0.645), one of which matched the topology of the tree produced by the neighbor-joining method (Fig. 2). Strict-consensus analysis showed general agreement among topologies of the 19 maximum-parsimony trees, with 57% (16 of 28) of bifurcations present in all 19 trees (Fig. 2). Most rearrangements among the 19 trees were found within the East Bay clade, results consistent with the greater genetic diversity detected for this population (Table 2). Parsimony and neighbor-joining analyses placed all subpopulations within clades that were consistent with their geographic populations, except that neighbor-joining analysis placed two subpopulations from the East Bay (EB3 and EB4) in a clade that diverged basal to the divergence of the main East Bay clade (Fig. 2). Parsimony analysis suggested a slightly different position for these two subpopulations. Eleven of the 19 maximum-parsimony trees (58%) placed the EB3-EB4 clade as a sister clade to the main East Bay clade (topology not shown). Lineages leading to the clades of all four populations were supported by bootstrap and jackknife percentages >50%. As seen with the initial cluster analysis, parsimony and neighbor-joining analyses of subpopulations indicated that the East Bay population was the first to diverge, followed by the North Bay, then the Salmon Creek and Peninsula populations (Fig. 2). Neighbor-joining analysis revealed that subpopulations within the East Bay, North Bay, and Peninsula populations had undergone significant differentiation since their divergence, while the subpopulations of Salmon Creek had undergone about half as much differentiation (mean genetic distance of subpopulations from the point of divergence was 12.4, 12.7, 6.8, and 11.4 for the East Bay, North Bay, Salmon Creek, and Peninsula populations, respectively). East Bay and Peninsula subpopulations had greater variation in the extent of differentiation than did the subpopulations of the North Bay and Salmon Creek (SD = 6.1, 1.8, 0.7, and 4.8 for the East Bay, North Bay, Salmon Creek, and Peninsula populations, respectively). A few of the subpopulations within the East Bay and Peninsula populations had particularly strong differentiation (e.g., EB4, EB8, and PN8; Fig. 2).

TABLE 2. Percentage of polymorphic loci and number of population-specific bands resolved using five 3′-anchored ISSR primers for geographic populations of *Dirca occidentalis*.

Population(s)	Percentage of polymorphic loci	Total no. loci	No. samples	No. population-specific loci
East Bay	30	419	60	40
North Bay	19	367	43	16
Salmon Creek	10	344	16	15
Peninsula	24	382	59	17
Species-wide	34	474	178	—
All samples, including outgroup	79	565	181	—

Notes: Results for East Bay, North Bay, Salmon Creek, and Peninsula describe the polymorphisms found within each of these populations. Species-wide results reflect polymorphisms among the four populations, and results for all samples include loci from the outgroup, *D. palustris*. Allele profiles for populations were generated from ≥50% consensus of allele presence or absence across all samples for each population. The number of samples evaluated for each population represented the relative size of the population, an approach that helped facilitate an accurate comparison of polymorphisms and specific loci (Hoel, 1966).

Parsimony and neighbor-joining analyses of population consensus profiles confirmed genetic separation of the four populations of *D. occidentalis* and verified the order of divergence shown by analyses with individual and subpopulation profiles. Wagner parsimony analysis at the population level resulted in two most-parsimonious trees requiring 336 steps (CI = 0.958, RI = 0.536). Consensus analysis showed that the clade formed by the Salmon Creek and Peninsula populations was present in both maximum-parsimony trees (Fig. 3). The topology of one of the trees matched the dendrogram produced by the neighbor-joining method (Fig. 3), and this topology was supported by bootstrap and jackknife percentages >50% (Fig. 3). Neighbor-joining analysis of the four populations indicated that the North Bay population was the most differentiated from its point of divergence (longest branch length; Fig. 3), the Peninsula population was the most evolved of the four populations (least similar to the genotypic makeup of their common ancestor), and the East Bay population was the least evolved (i.e., most similar to the genotypic makeup of their common ancestor).

The Mantel test for matrix correspondence revealed a positive correlation between geographic and genetic distances among all subpopulations of *D. occidentalis* ($r_{xy} = 0.29$, $P \leq 0.001$). Regression analyses showed that, while a positive linear relationship existed across all pairwise distances (Fig. 4, red line), a stronger linear relationship between geographic and genetic distances was evident for subpopulations ≤70 km apart (Fig. 4, blue line). GenaAlEx analyses of spatial autocorrelation with multiple distance classes further resolved the spatial genetic structure of *D. occidentalis.* Evaluation of multiple distance classes set at 10-km increments revealed that genetic distances increased with geographic distances between 0 and 70 km. Permutation and bootstrap tests rejected the null hypothesis of no spatial structure for each 10-km increment from 0 to 70 km (Fig. 5, correlation values [r_c] were outside permutation null confidence interval, and bootstrap confidence intervals [error bars] did not straddle $r_c = 0$) and retained the null hypothesis for increments >70 km (Fig. 5, r_c within permutation null confidence interval, and bootstrap confidence intervals [error bars] straddled $r_c = 0$). Analysis of multiple distance classes at 1-km increments refined the autocorrelation model by showing the limit of genetic structure to be 66 km according to bootstrap (Fig. 5, orange vertical line) and 75 km according to permutation (Fig. 5, red vertical line). In the plot of autocorrelation based on 1-km increments (Fig. 5, green line), the strongest correlations between geographic and genetic distance were for subpopulations ≤3 km apart ($r_c = 0.50$, 0.48, and 0.47 at distances of 1, 2, and 3 km, respectively), then a sharp decline followed by a plateau for subpopulations 4–38 km apart (mean $r_c = 0.37$). Another sharp decline in correlation was shown for geographic distances of 38–66 km, and correlations were not significant at distances ≥66 km according to bootstrap analysis (≥75 km by permutation).

DISCUSSION

An important concept common to phylogenetics and population genetics is that divergence and differentiation of isolated populations exist over a range from genotypes indistinguishable

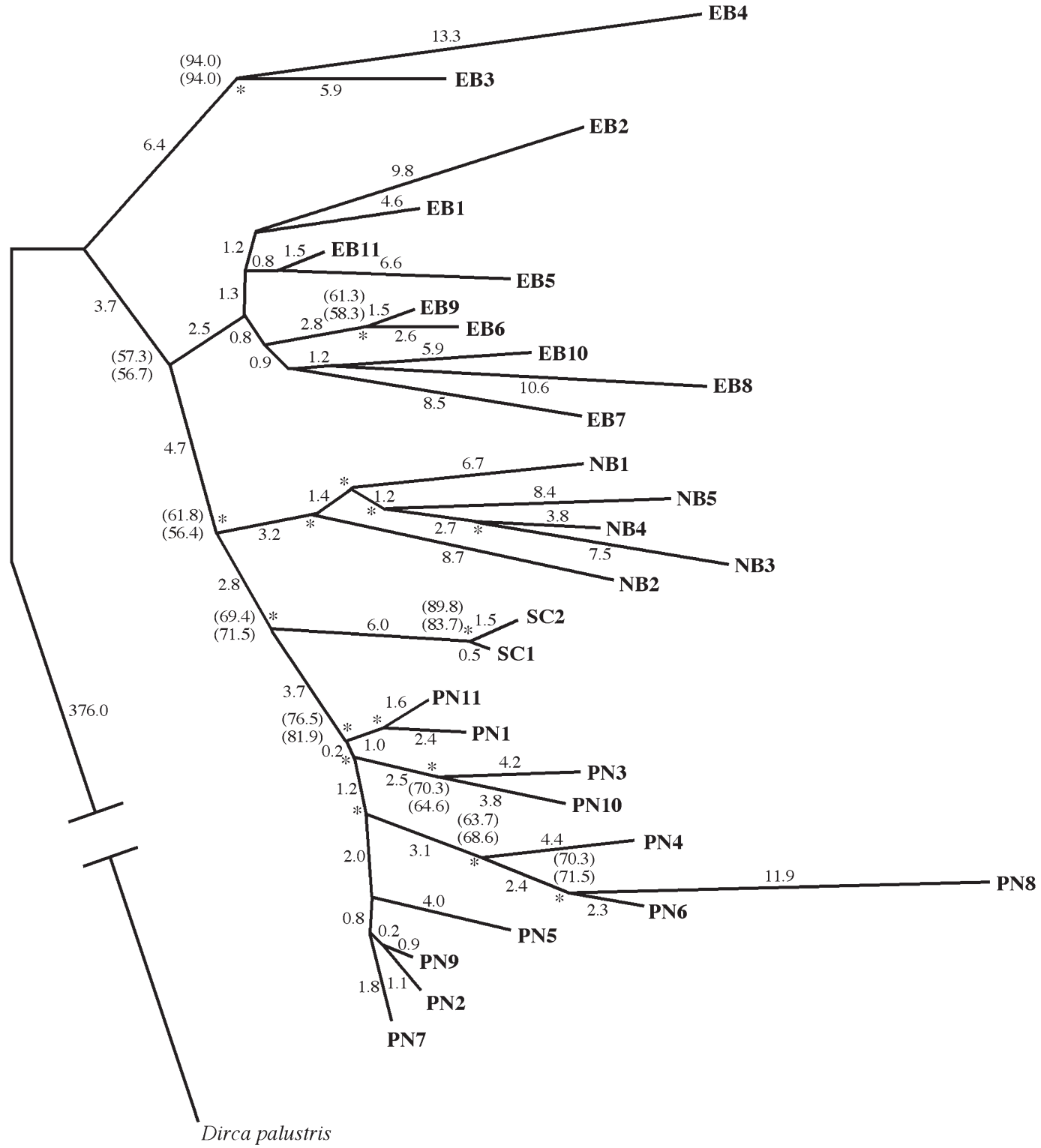

Fig. 2. ISSR neighbor-joining dendrogram showing the inferred phylogenetic relationship among 29 geographically defined subpopulations of *Dirca occidentalis*. The first two letters of each name indicate the population of origin (EB = East Bay, NB = North Bay, SC = Salmon Creek, PN = Peninsula), which is followed by the subpopulation number. Numbers at the middle of each branch indicate relative branch lengths; numbers in parentheses are the bootstrap (top) and jackknife (bottom) percentages, included when both values were greater than 50%. *Dirca palustris*, the species most closely related to *D. occidentalis* (Schrader and Graves, 2004b), was used as the outgroup to establish ancestral-character polarity of ISSR banding patterns. The topology of the dendrogram represents one of 19 most-parsimonious trees requiring 541 steps according to Wagner parsimony analysis (CI = 0.723, RI = 0.645). Clades that appeared in all 19 most-parsimonious trees are indicated with asterisks (*).

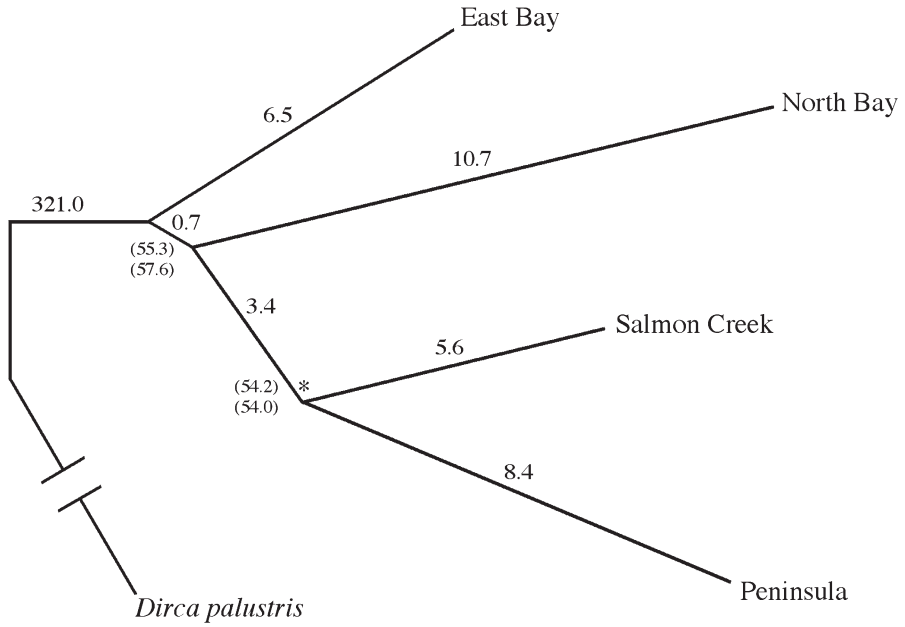

Fig. 3. ISSR neighbor-joining dendrogram showing the inferred phylogenetic relationship among the four populations of *Dirca occidentalis*. Numbers indicate relative branch lengths; numbers in parentheses are the bootstrap (top) and jackknife (bottom) percentages. *Dirca palustris*, the species most closely related to *D. occidentalis* (Schrader and Graves, 2004b), was used as the outgroup to establish ancestral-character polarity of ISSR banding patterns. The topology of the dendrogram represents one of two most-parsimonious trees that required 336 steps according to Wagner parsimony analysis (CI = 0.958, RI = 0.536). The clade that appeared in both most-parsimonious trees is indicated with an asterisk (*).

from other populations to genotypes divergent enough to be considered separate species (Simpson, 1961; Nelson and Platnick, 1981; Russell et al., 2007). The four geographically defined populations of *D. occidentalis* (Fig. 1) have diverged and differentiated, but the degree of differentiation is small relative to that normally considered requisite for speciation. The populations have been reproductively isolated long enough that genetic differences can be resolved by hypervariable, ISSR markers (Table 2, Figs. 2 and 3), but our cursory observations of *D. occidentalis* over several years have revealed no consistent morphological differences among the four populations. While the level of divergence is less than the amount needed for subspecific classification of the populations (i.e., no ecological specialization or morphological means of distinction; Shetler et al., 1973), patterns of genetic variation among and within the populations shed light on the phylogenetic history and phylogeography of this rare, endemic species.

Evidence from the inferred phylogeny and the percentage of polymorphism indicates that the East Bay population is the most genetically diverse and the most similar to the common ancestor of the four populations (Table 2, Fig. 3), suggesting that *D. occidentalis* invaded the San Francisco Bay area from the east. The hypothesis that *D. occidentalis* invaded the area from the east is consistent with paleobotanical reports that document the presence of ancestral members of the Thymelaeaceae in the late Eocene flora of central Colorado (MacGinitie, 1953; Graham 1993). More recently, researchers evaluating populations of *Dirca* L. near the western extreme of *D. palustris* have identified a disjunct population in Kansas that possesses morphological characters that appear to be intermediate among the three disjunct species of *Dirca*, *D. palustris* (eastern United States), *Dirca mexicana* Nesom and Mayfield (northeastern Mexico), and *D. occidentalis* (Floden and Mayfield, 2006). Taken as a whole, this evidence suggests that the ancestral center for modern species of *Dirca* was the central United States (Ronquist, 1997). Climatic changes and fluctuations, along with geological disruptions in the central and western United States since the Eocene, likely brought both migration and isolation of *Dirca* elements leading to the present disjunct distribution of the genus (Graham, 1993; Schrader and Graves, 2004b). Migration into western California, followed by the occurrence of a dryer climate in the Basin and Range Province since the end of the Pliocene (Axelrod, 1986; Graham 1993), has established *D. occidentalis* in its present-day refuge surrounding the San Francisco Bay.

One aspect of the phylogenetic results that is somewhat paradoxical is that the Salmon Creek population is more genetically similar to the Peninsula population than to the North Bay population (Figs. 2, 3), even though the North Bay is geographically between Salmon Creek and the Peninsula (Fig. 1). While long-distance dispersal of germplasm from Salmon Creek to the Peninsula, bypassing the North Bay, might explain this phylogeographic structure within *D. occidentalis*, there is little evidence to support this. Only rarely have we observed evidence of possible frugivore activity during development of drupes of *D. occidentalis*, there have been no definitive reports of extant or extinct frugivores, and there are no specialized means of seed dispersal for the species (Johnson, 1994). We considered the possible impact of use and cultivation of *D. occidentalis* by Native Americans on the present phylogeographic structure but found no record of the species in ethnobotanical literature pertaining to known local tribes (Bocek, 1984; Bocek and Reese, 1992). A more likely explanation for this phylogeographic pattern can be found in the climatic and geological history of the San Francisco Bay area. While the coarse topography of the San Francisco Bay region was formed in the Mesozoic and

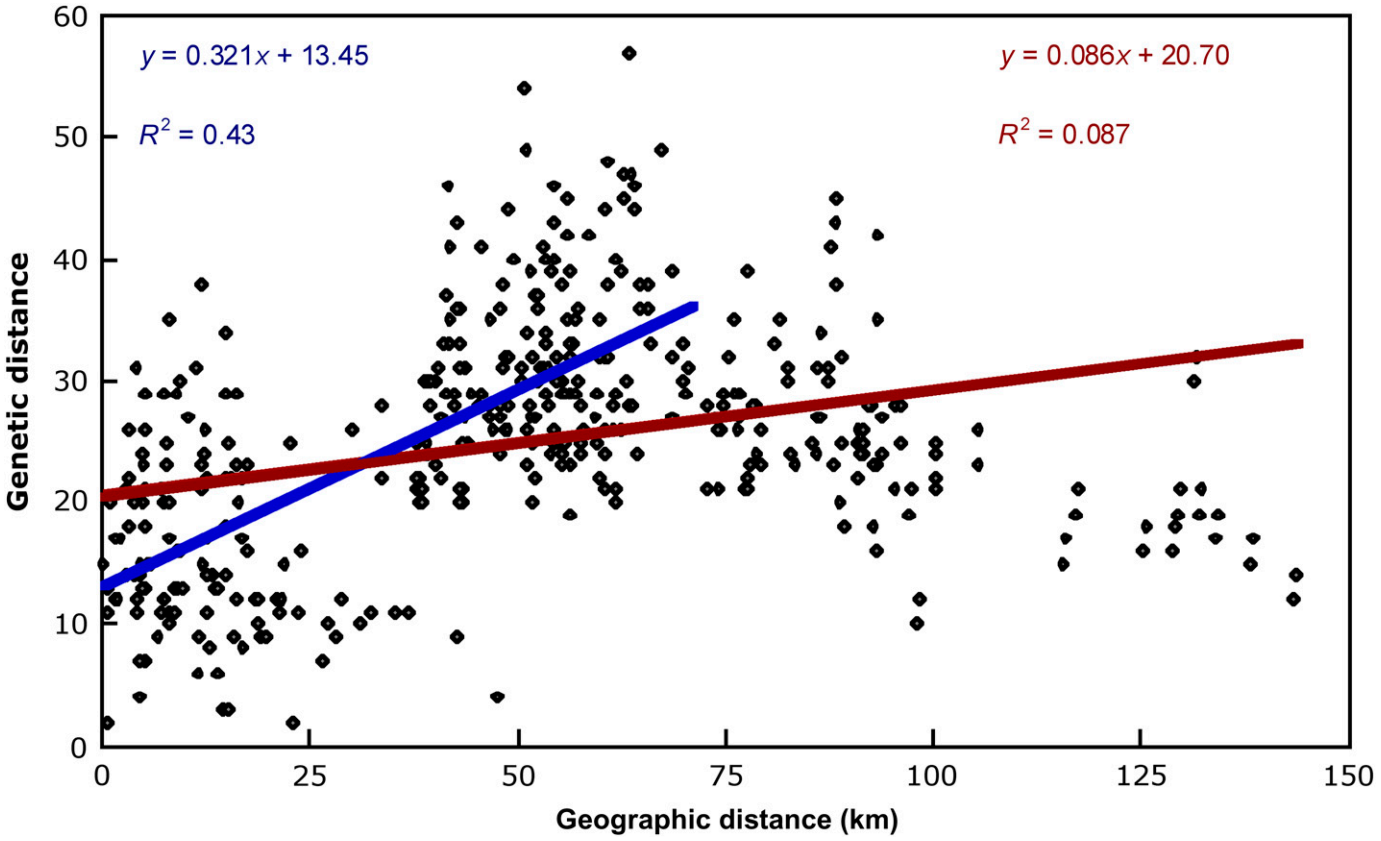

Fig. 4. Genetic distance between subpopulations of *Dirca occidentalis* (*y*-axis) as a function of geographic distance (*x*-axis). Symbols represent individual pairwise relationships. The red and blue lines represent linear regressions of the full data set and a subset comparing subpopulations ≤70 km apart, respectively.

early Cenozoic eras by the dynamic movement of geological plates and by erosion, the existence of the bay is more recent and more directly a product of climate fluctuations (Howard, 1979; Harden, 1998; Sloan, 2006). During glacial periods, global temperatures were cool, worldwide sea levels low, and the present-day San Francisco Bay was dry land with the exception of rivers and streams that carved channels through the lowland plain. During interglacial periods, temperatures were warmer, sea levels higher, and the bay was an estuary much like it is today. At the last interglacial, the bay was larger than it is today, and at the peak of the last major glacial episode (≈20 000 yr ago [ya]), the sea level is believed to have been 120 m lower than at present, the bay was dry, and the Pacific coastline in the San Francisco area was ≈32 km west of present-day beaches (Harden, 1998; Sloan, 2006). The cooler temperatures and continuous land across the bay area during the last glacial period probably resulted in panmixis of *D. occidentalis* across the region. As temperatures began to rise with the development of the present interglacial period, heat and increased competition from thermophilic plants in the lowland plains would have rendered the habitat less hospitable for *D. occidentalis*, and the populations would have begun to segregate to their present geographical limits.

Results of our phylogenetic analyses suggest that, before the rising sea level moved the Pacific coast to its present location, development of the Mediterranean climate and interspecies competition brought first the isolation of the East Bay population, then the North Bay population, then finally the Salmon Creek and Peninsula populations, which had maintained gene flow throughout the western coastal plain after becoming isolated from the North Bay population. Fog may have been another factor that contributed to the longevity of gene flow across the historical western coastal plain between the Salmon Creek and Peninsula populations. Botanists have suggested that contemporary occurrence patterns of fog in the San Francisco Bay Area during summer coincide with locations where *D. occidentalis* is prevalent (Johnson, 1994). With the historic coastline extending further west, it is plausible that fog banks of ≈20 000 ya failed to reach critical areas between the East Bay, North Bay, and Salmon Creek populations, but did nourish the continuous strip of land between the Salmon Creek and Peninsula. While our results indicate that climate change and ecological forces likely brought the isolation of the four populations in the order indicated, inundation of the bay and western coastal plain, which reached its present limit by ≈4000 ya (Harden, 1998), has ensured the isolation of populations northwest, south, and east of the bay. More broadly, our results illustrate the importance of examining geological and climatic histories when reconstructing the geographic and natural history of plant species. Due to the intriguing geology and topography of land near the San Francisco Bay and to numerous core samples of sediment extracted in preparation for construction of bridges that span the bay, geological and climatic data for the area abound (Howard, 1979; Harden, 1998; Sloan, 2006). Without this rich base of information, explanations for much of the phylogeographic structure of *D. occidentalis* would be elusive. This study therefore illustrates

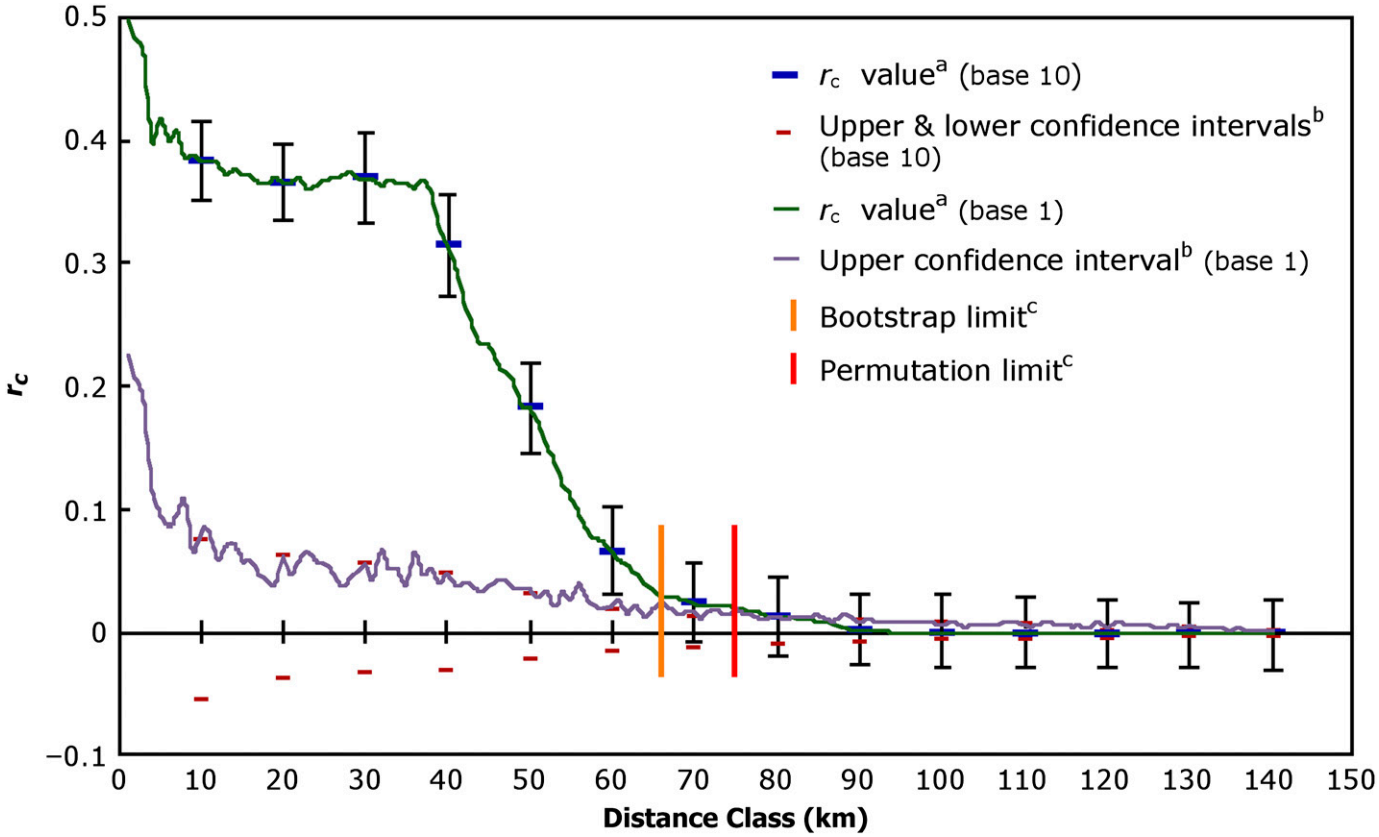

Fig. 5. Spatial genetic correlation (r_c) of *Dirca occidentalis* based on autocorrelation analyses of subpopulation consensus genotypes at multiple distance classes. [a]Blue symbols and green curve represent correlation (r_c) values from multiple-distance-class analyses with base class sizes of 10 and 1 km, respectively. Error bars for the blue symbols indicate 95% confidence intervals at base class sizes of 10 km as determined by 1000 bootstrap resamplings. When the bootstrap confidence interval does not straddle $r_c = 0$, significant genetic structure is inferred. [b]Red symbols represent the upper and lower 95% confidence intervals about the null hypothesis of a random distribution of genotypes according to 999 permutations at base class sizes of 10 km, and the violet curve represents the upper 95% interval at base class sizes of 1 km. [c]Orange and red vertical lines indicate the limits of spatial genetic structure as determined by bootstrap and random-permutation tests, respectively.

the value of data on geological and climatic history as botanists resolve questions pertaining to plant distributions and phylogeography.

Results of spatial-genetic analyses support our conclusion that the four populations of *D. occidentalis* are genetically isolated and help to characterize the genetic structure of *D. occidentalis*. Both the Mantel test and the analyses of spatial autocorrelation indicate that the limit of genetic structure in *D. occidentalis* is ≈70 km (Figs. 4 and 5), a distance that coincides with the mean geographical distance among subpopulations in separate populations (68.1 km). The sharp decline in autocorrelation for geographical distances between 38 and 66 km (Fig. 5) reflects the portion of the analysis where within-population and among-population comparisons overlap, and the limit of autocorrelation (66 km by bootstrap, 75 by permutation; Fig. 5) reflects the genetic isolation (lack of genetic structure) for comparisons among subpopulations in separate populations. Essentially, these results indicate that spatial genetic structure exists only within populations, where genetic distance increases with geographic distance.

Results of the autocorrelation analysis for geographic distances ≤38 km (almost all of which were within-population comparisons) help to characterize the interaction among four important factors that affect reproduction of *D. occidentalis*. The very strong autocorrelation for subpopulations ≤1 km apart ($r_c = 0.50$) is consistent with the observation that *D. occidentalis* seems to lack an effective means of seed dispersal (Johnson, 1994). Most *D. occidentalis* occupy positions on sloped land, and the only apparent means of dispersal is newly abscised fruits rolling down slope short distances before becoming embedded in surface organic matter. Genetic dispersal by this means would correlate well with geographic distances ≤1 km. While it is probable that clonal reproduction by rhizomes (Graves, 2004) contributes to the genetic structure of *D. occidentalis*, it seems most likely that this contribution is within distances of approximately 5 m because we sampled both resolved and unresolved genotypes within this distance of one another. It is doubtful that clonal reproduction contributes to genetic structure at broader levels of the species distribution. Subpopulations typically are separated by several kilometers, and none of the sets of unresolved genotypes included plants from multiple subpopulations. Further research should focus on the frequency of clonal regeneration of *D. occidentalis* and its impact on the genetic diversity and fine structure within the species.

Other factors that might affect genetic structure within populations are related to pollination. Floral morphology of *D. occidentalis* is of the type that favors visits by pollinators, and pollen is mucilaginous and not readily released in wind.

We have observed two potential pollinators visiting flowers of *D. occidentalis*, Anna's hummingbird (*Calypte anna* Lesson) and honeybee (*Apis mellifera* L.). Although both the historical significance of these potential pollinators and the distance they might travel to obtain nectar during winter when *D. occidentalis* is in bloom are uncertain, the range of travel of bees and hummingbirds may be reflected in the strong autocorrelation for distances ≤3 km and the sharp decline and plateau for distances between 4 and 38 km (Fig. 5). *Apis mellifera* rarely forages >6 km from the hive and normally forages at shorter distances (mean ≈1 km) when nectar sources are abundant (Beekman and Ratnieks, 2000). It is conceivable that bees, or some other insect, cover a relatively small area (≈3 km) in search of nectar and that their interaction with flowers of *D. occidentalis* is a major contributor to the strong genetic structure among subpopulations within 3 km of each other; the sharp decline in autocorrelation at 4 km may signal the limit of insect pollinators. The plateau seen in the autocorrelation for distances between 4 and 38 km may reflect the interaction of *D. occidentalis* with the hummingbird or some other medium-distance pollinator. It is reasonable to postulate that the probability of visitation by a pollinator of this sort would decrease with geographic distance and that the probability of such a pollinator making a trip across the bay or between the Salmon Creek and North Bay populations would be low, a hypothesis that could explain the genetic isolation of the four populations. Ornithologists have confirmed that, while Anna's hummingbird does not have a true migration habit, it does travel over moderate distances and to higher and lower elevations with seasonal changes in food sources and during movements to and from breeding territories (Johnsgard, 1983; Russell, 1996). The timing of seasonal travel of Anna's hummingbird likely coincides with anthesis of *D. occidentalis* (Pitelka, 1951; Russell, 1996), and the distances traveled by hummingbirds during these times may help to explain the genetic structure of *D. occidentalis*. Recent research reveals that *D. occidentalis* can self-pollinate but suggests that the impact on population genetic structure probably is low. Fruit set is reduced by 75% among self-pollinated flowers compared with fruit set among open-pollinated flowers, and seeds that result from self-pollination germinate at a reduced rate (W. Graves and J. Schrader, unpublished data).

The limited geographic range of *D. occidentalis* and its capacity to self-pollinate and to reproduce asexually from rhizomes have generated concern that genetic diversity of this species might be low (Graves, 2004), a condition that could signify poor ecological fitness due to inbreeding depression and a reduced capacity of populations to evolve in response to environmental changes (Reed and Frankham, 2003). Our findings indicate that genetic diversity is not critically low across the species as a whole (34% ISSR polymorphism), but is low within the North Bay and Salmon Creek populations, which showed ISSR polymorphism of 19 and 10%, respectively (Table 2). The low genetic diversity, sparseness of plants (Howell, 1970; Best et al., 1990), and small geographic size of these two populations underscore the vulnerability of *D. occidentalis* northwest of the San Francisco Bay. The Salmon Creek population should be considered extremely vulnerable. With mature plants numbering ≈50, a range no greater than 50 hectares, and low genetic diversity (ISSR polymorphism of 10%), this population has signs of poor ecological fitness and an elevated risk of extinction (Reed and Frankham, 2003; Thompson, 2005). The greater geographic size, plant density, and genetic diversity of the East Bay and Peninsula populations indicate that they are less vulnerable than the North Bay and Salmon Creek populations. Clonal regeneration and self-pollination likely play roles in sustaining *D. occidentalis* (Graves, 2004, 2005), but results of this study and of our research examining self-pollination (unpublished data) suggest impacts are modest and localized. Future research should assess the consequences of asexual spread and self-pollination in the North Bay population, within which ISSRs failed to resolve 10 of the 43 sampled plants, and in the Salmon Creek population, which contains the fewest plants on the smallest land area among the four populations.

Our main goals were to characterize the genetic structure and phylogeography of *D. occidentalis*, to determine if its disjunct populations are genetically isolated, and to reconstruct the geographical and natural history of this rare endemic shrub. While ITS-sequence markers have been unable to resolve genetic variation within *D. occidentalis* (Schrader and Graves, 2004b) and many other plant species (Schaal et al., 1998; Pelser et al., 2003), ISSR markers resolved variation suitable for analyses at the interface of phylogenetics and population genetics. Our phylogenetic and spatial analyses confirm that the four populations of *D. occidentalis* are genetically isolated and have undergone divergence, but the level of divergence is not sufficient to divide the species taxonomically. The genetic structure within and among the populations of *D. occidentalis* suggests an interaction of four main factors: limited seed dispersal, clonal regeneration, distances traveled by pollinators, and genetic isolation of the four populations. While the genetic diversity of the East Bay and Peninsula populations appears to be relatively robust, diversity within the North Bay and Salmon Creek populations is low, indicating poor ecological fitness and risk of decline. Our results reveal an unexpected order of isolation and divergence among the four populations of *D. occidentalis* in which the populations at the northern and southern extremes of the species distribution (Salmon Creek and Peninsula populations) were the last to diverge. An explanation for this unusual event lies in the dynamic geological and climatic history of the Bay Area, which illustrates the relevance of climatic and geological data when resolving the geographical and natural history of plant species.

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