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Investigating species boundaries in the Giliopsis group of *Ipomopsis* (Polemoniaceae): Strong discordance among molecular and morphological markers[1]

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As a first step in elucidating mechanisms of speciation in the Giliopsis group of *Ipomopsis* (Polemoniaceae), we examined patterns of morphological and genetic differentiation and crossability. This group comprises three species that diverged very recently: two perennials, *I. guttata* and *I. tenuifolia*, and one annual, *I. effusa*. Analysis of phenotypic variation established that the three species are distinct for floral characters, and this differentiation is maintained in a locality containing both perennial species. Next, we assessed the genealogical relationships with AFLPs. All sampled individuals of *I. effusa* clustered together, a result in accord with its genetic isolation. The perennials, which retain interfertility, were not resolved as sister taxa. Rather, individuals sampled from the single *I. guttata* population that is sympatric with *I. tenuifolia* were genetically more similar to *I. tenuifolia* samples than they were to conspecifics. This pattern may be due to substantial introgression of *I. tenuifolia* genomic regions that do not contribute to floral phenotype in *I. guttata*. Our result adds to mounting evidence that plant species, as defined by morphological characters, are often not genomically cohesive. Taken together, our data warrant caution in delimiting species with genetic markers alone, and, importantly, suggest that selection on species-diagnostic morphological characters can be sufficiently strong to counteract extensive gene flow.

Key words: AFLP; Giliopsis group; introgression; *Ipomopsis*; Polemoniaceae; selection; species; taxonomy.

Study of the origin, maintenance, and circumscription of species diversity is fundamental to evolutionary biology (Coyne and Orr, 2004). First, species delimitation, when conducted carefully, leads to the construction of sound taxonomies that facilitate conservation efforts (Isaac et al., 2004). Second, any conclusions about the processes that are most important to speciation are ultimately dependent upon how one circumscribes species in practice. Most plant species are named on the basis of morphological discontinuity in one or more characters that separates them from close allies (Michener, 1970; Cronquist, 1988). Thus, the student of plant speciation is interested in describing the historical events and ecological processes that were involved in the origin of this discontinuity. Consequently, building a historical hypothesis of relations among closely related species is a critical, but challenging, first step in the study of speciation, in part, because it allows one to infer the relative timing of divergences in species-diagnostic characters. In addition, analysis of species differences is most informative between sister pairs, and therefore, identifying morphological shifts that are intrinsic to speciation requires fine-scale phylogenetic resolution. Here, we present genetic, morphologic, and crossability data that bear on the species level taxonomy and phylogeny of the Giliopsis group of *Ipomopsis*. These data serve as a starting point for studying speciation and ongoing evolution within this group, provide important insight into the biology of a rare species, and highlight several general challenges to assessing and delimiting plant species diversity.

Many congeneric plant species are separated on the basis of floral characters that appear to be correlated with adaptation to different suites of pollinators (Grant, 1949). *Ipomopsis* (Polemoniaceae) is a classic example of such a genus; interspecific variation in floral form is remarkable and members of the group attract a wide array of pollinators, e.g., birds, beetles, bees, bombyliids, and butterflies (Grant and Grant, 1965). Within *Ipomopsis*, the Giliopsis group comprises three species, *I. effusa* (A. Gray) Moran, *I. guttata* (A. Gray) Moran, and *I. tenuifolia* (A. Gray) V. E. Grant (Moran, 1977). The three forms are separated primarily by floral characters, and our field observations indicate that each is serviced by a distinct suite of pollinators: *I. effusa*, bees; *I. guttata*, bombyliid flies and lepidopterans; and *I. tenuifolia*, hummingbirds. Thus, our observations suggest that each recognized species fulfills a unique ecological role and that divergent selection mediated by pollinators has caused their divergence.

The coupling of floral character divergence and pollination mode in the Giliopsis group resembles the pattern seen in the *I. aggregata* complex. Extensive work in this complex has greatly advanced our understanding of the role of pollinators in shaping floral variation within lineages (e.g., Campbell et al., 1991; Mitchell and Waser, 1992) and maintaining it among taxa (e.g., Campbell, 2004). While interspecific hybrids often occur at zones of contact between species of the *I. aggregata* complex (e.g., Grant and Wilken, 1988), intermediates between Giliopsis species are absent despite range overlap. Thus, natural barriers to gene exchange are apparently more complete in this group. In general, study of Giliopsis complements the classic work in the *I. aggregata* complex. For example, hummingbird pollination has most likely arisen independently within the *I. aggregata* complex and within Giliopsis, and ongoing work in these two groups will enable comparative analyses of the

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genetic and morphologic changes that accompany adaptation to hummingbird pollination.

The three Giliopsis species are distributed across the northern half of Baja California and into Alta California. *Ipomopsis guttata* is restricted to two small, disjunct regions (ca. 800–1600 m a.s.l.): one in the Sierra Juarez (SJ, the northern population system) and the other in the Sierra San Pedro Martir (SSPM, the southern population system). *Ipomopsis guttata* can be locally abundant in these areas, although it is clearly a rare taxon that appears to be specialized to chamise (*Adenostoma* spp.) dominated chaparral habitat (Moran, 1977). *Ipomopsis tenuifolia* has a much greater ecological amplitude, with populations occupying a variety of altitudes and habitat types, from the upper reaches of the SJ and SSPM (up to 2400 m a.s.l.) to the desert floor (100 m a.s.l.; Moran, 1977), and ranging from southern Alta California to the cardon cactus forests of north central Baja California. *Ipomopsis guttata* and *I. tenuifolia* are in direct sympatry in one location in the SSPM, and given that the range of *I. tenuifolia* encompasses that of *I. guttata*, they probably co-occur in other, yet undiscovered localities. The third species, *I. effusa*, typically occurs as extensive populations carpeting pine meadows in the higher reaches of the SJ and SSPM (875–2600 m a.s.l.), but also appears sporadically along watercourses at substantially lower elevations (Henrickson, 1987). All three species have peak flowering times in early to midsummer, but, depending on rainfall, can flower from February to December. *Ipomopsis effusa* is an annual, while the other two species are suffrutescent perennials, dying back to a woody base during drier periods. All three species occur in regions that undergo periodic fires (Minnich and Franco-Vizcaino, 1998), and they are often collected in flower after recent burns.

These three species were originally described as members of *Loeselia* (Gray, 1876, 1885) a genus that, like the Giliopsis group, has zygomorphic corollas, an uncommon feature in the Polemoniaceae. On cytotypic ($N = 7$) and morphologic evidence, Grant (1956) transferred the two perennial taxa to *Ipomopsis* and placed *I. guttata* in synonymy under *I. tenuifolia*. In addition, he moved *I. effusa* to *Gilia*. Moran (1977) later moved *G. effusa* to *Ipomopsis* and resurrected *I. guttata*. While the circumscription of *Ipomopsis* continues to be problematic (Grant, 1956; J. M. Porter, Rancho Santa Ana Botanic Garden; L. A. Johnson, Brigham Young University; D. H. Wilken, Santa Barbara Botanic Garden; unpublished manuscript), it is clear that these three species form a clade. However, the relationships among the three forms cannot be resolved with current molecular data (*trnL/F* and ITS sequences; J. M. Porter, L. A. Johnson, D. H. Wilken, unpublished manuscript), and detailed analyses of morphologic and genetic variation within the group are lacking.

While Moran (1977), in the most recent taxonomic treatment of the group, concluded that the three species are readily separated, he did not present quantitative evidence supporting this conclusion. Because there is substantial intraspecific variation in characters that are used to separate the three species, particularly within *I. guttata* and *I. tenuifolia*, we evaluate their distinctness with multivariate ordination of phenotypic variation. This analysis enables a more objective discussion of species delimitation in this group.

In addition, we clarify the genealogical relationships of these closely allied species. In particular, given that we are using *I. guttata* and *I. tenuifolia* to study the genetic basis of adaptive divergence and speciation, we are interested in determining which two of these three species coalesce to the exclusion of the third. Because all current evidence suggests that the three species share a very recent common ancestry, we employ AFLP markers because they are known to provide phylogenetic signal at lower taxonomic levels, are cost effective, are reasonably reproducible and provide a broad, genome-wide sample (Koopman, 2005). This latter feature is critical given that inferences of relationships among closely related plant species may vary depending on marker choice (e.g., Doyle, 1992). Rokas et al. (2003) suggested that roughly 20 genes should be sufficient to resolve infrageneric relationships robustly, but attempts to resolve the chimp–gorilla–human trichotomy demonstrate that even with Herculean effort, clear answers can remain elusive (Rokas and Carroll, 2006). Indeed, recent work with *Drosophila* reveals that phylogenetic resolution among congeneric species can be equivocal even when whole-genome sequences are analyzed (Pollard et al., 2006). In the case of Giliopsis, AFLPs provide an efficient, genomic-average approach to evaluating the relationships of the three recognized species.

As with many closely related plant species, the two perennial taxa can be crossed to yield fertile F1 plants. At the sympatric locality in SSPM, there is some visible evidence of gene exchange. To assess the potential for interspecific gene flow in nature, we quantified interfertility with controlled crosses of cultivated individuals from multiple populations of each species. Because gene flow and genealogy are interdependent (Avise and Wollenberg, 1997), these data enable a clearer interpretation of our genealogical results. In addition, while crossability need not be correlated with relatedness, such data serve as an interesting complement to other measures of phylogenetic affinity.

MATERIALS AND METHODS

Morphological analysis—Patterns of variation in morphological and life history traits (8 continuous, 3 categorical; Table 1) were investigated with principal component analysis using the program JMP version 5 (SAS Institute, 1989–2002), and the first three principal components of variation were plotted using the program SigmaPlot 9.0 (Systat Software, 2004). Measurements were taken from greenhouse-grown plants (*I. effusa*, $N = 12$; *I. tenuifolia*, $N = 21$); *I. guttata* (from Sierra Juarez [SJ], $N = 30$); *I. guttata* (from Sierra San Pedro Martir [SSPM], $N = 9$). In addition, we included an experimental population of F1 hybrids ($N = 30$) between *I. tenuifolia* and *I. guttata* in the analysis to allow comparisons with the SSPM population of *I. guttata*, which shows phenotypic evidence of hybridization with sympatric *I. tenuifolia*. Plants were grown under artificially long days (16 h) to stimulate flowering. In most cases, morphological data for individual plants represent a mean of two measurements. To quantify nectar volume, we removed flowers on the second day after anthesis and cut them just above the calyx. Nectar was spun down from inverted calyxes/corolla tube bases in a centrifuge, drawn into a 5-μL disposable pipette, and the length (in millimeters) of the extract in the pipette was measured. In all cases, individual *I. effusa* flowers had a very small but visible amount of nectar, which could not be readily drawn into the pipette. These nectar amounts were assumed to be equal to 1 mm. Anthocyanin was extracted from petal lobe tissue following Wilken (1982). Absorbance (scaled by sample mass) at 515 nm was measured with a spectrophotometer, and this quantity was used as the anthocyanin concentration metric.

Crossability analysis—To estimate the potential for natural gene exchange among the species, we performed interspecific crosses in the glasshouse. For comparison to these interspecific crosses, we also made crosses within species. We used individuals from at least two populations of each species, and pollen parents were drawn from a pool of 13, 20, and nine individuals, for *I. effusa*, *I. guttata*, and *I. tenuifolia*, respectively. While all species are self-incompatible, to avoid autogamous fruit set, we removed the entire androecium from flowers selected for pollination prior to pollen deposition. Two measures of interfertility were measured: (1) the ability to set F1 seed (on pure parents) and (2) F1

TABLE 1. Character means/states of phenotypes used for principal component analaysis. For interpretation of anthocyanin and nectar values, see Materials and Methods. *Ipomopsis effusa* (*N* = 12); *I. guttata* (Sierra Juarez [SJ]; *N* = 30); *I. guttata* (Sierra San Pedro Martir [SSPM]; *N* = 9); *I. tenuifolia* (*N* = 21).

Character	Species (region)			
	I. effusa	*I. guttata* (SJ)	*I. guttata* (SSPM)	*I. tenuifolia*
Midcauline leaf length, mm (±SE)	18.48 (0.46)	17.50 (0.57)	15.00 (0.85)	17.23 (0.99)
Stamen length, mm (±SE)	10.80 (0.31)	17.24 (0.37)	18.92 (1.35)	32.30 (0.51)
Pistil length, mm (±SE)	12.18 (0.12)	18.11 (0.39)	17.73 (1.50)	32.87 (0.79)
Corolla tube length, mm (±SE)	4.30 (0.12)	9.17 (0.17)	11.16 (1.04)	18.59 (0.43)
Petal lobe anthocyanin content (±SE)	23.44 (0.88)	4.74 (0.27)	16.89 (1.49)	36.71 (1.49)
Nectar quantity, mm (±SE)	1 (0.00)[a]	25.92 (3.19)	14.22 (3.37)	97.56 (8.81)
Corolla tube width at calyx, mm (±SE)	1.78 (0.07)	1.37 (0.02)	1.16 (0.02)	1.71 (0.03)
Corolla tube width at throat, mm (±SE)	1.80 (0.06)	1.75 (0.03)	1.57 (0.04)	2.89 (0.07)
Life history[b]	0	1	1	1
Petal lobe pigmentation[c]	0	1	1	0
Flower symmetry[d]	1	1	1	0

[a] Nectar quantity for *I. effusa* rounded up to 1.0 mm (see Materials and Methods); [b] 0 = annual, 1 = perennial; [c] 0 = uniform, 1 = speckled; [d] 0 = radial; 1 = bilateral (zygomorphic).

seed germinability. Seeds were stored at 4°C for 4–6 wk before germination trials. Seeds were planted in a potting mix of equal parts sand, soil, and gravel, which was kept wet for the 4 wk of the germination experiment. Fruit set results are reported as the proportion of pollinated flowers that set fruit. Data were pooled within each pairwise species combination, and in the case of crosses between perennial individuals, direction of cross. Because there were relatively few interspecific crosses involving *I. effusa*, data were simply pooled by pairwise species combination.

AFLP genotyping—Populations sampled for AFLP analysis are listed in Appendix 1. In total, 44 individuals were genotyped. Sampling was centered on the distributional area of *I. guttata* and *I. effusa* (Fig. 1). Populations from the northern and southern ends of the range of *I. tenuifolia* were not sampled. The utility of AFLPs vis-à-vis phylogenetic inference is limited to clades that coalesce fairly recently (Mueller and Wolfenbarger, 1999; Koopman, 2005). Several species outside of Giliopsis were also genotyped, but banding patterns were markedly different from those within the group. This result is in accord with nuclear and chloroplast markers, which reveal a long branch connecting Giliopsis to its sister lineage (J. M. Porter, Rancho Santa Ana Botanic Garden; L. A. Johnson, Brigham Young University; D. H. Wilken, Santa Barbara Botanic Garden; unpublished manuscript). Because the frequency of comigrating, nonhomologous bands is expected to increase as a positive function of phylogenetic distance (O'Hanlon and Peakall, 2000), taxa outside of Giliopsis were excluded.

Genomic DNAs were extracted from fresh or herbarium material of the 44 accessions using the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA). Individuals were genotyped following the protocol of Vos et al. (1995) with modifications described in Kim and Rieseberg (1999). The following primer pairs were used for selective amplification: Eatg (NED), H/Magt; Eacg (NED), H/Magt; Eact (FAM), H/Matc; Eatt (FAM), H/Magat; Eaca (PET), H/Matga; Eata (PET), H/Maaac; Eatc (VIC), H/Matc; Eacc (VIC), H/Magt. Amplified fragments were separated on an ABI 3730 (Applied Biosystems, Foster City, California, USA). Electropherograms were generated and analyzed using the program GeneMapper version 3.7 (Applied Biosystems) with default AFLP settings. Individuals were genotyped automatedly, which is arguably more objective (Meudt and Clarke, 2007), for all bands detected in the range of 50–450 bp (0 = band absent, 1 = band present; 2333 total markers, each treated as independent), and these data were converted to a NEXUS file. Absence/presence of markers in a few regions of individual electropherograms that were of low quality were scored as unknown (?) in the NEXUS file. Most significantly, the northern sample of *I. effusa* (EFTH), the DNA for which came from older, dried material, had poor reads from ca. 290 bp and longer for most primer pairs. Neighbor-joining trees were generated with the program PAUP* version 4.0b10 (Swofford, 2002), with both the standard distance option as well as the Nei–Li restriction site option. The balance between homologous vs. homoplastic shared absences determines whether it is more appropriate to use standard distances or the Nei–Li restriction site model. Unfortunately, this issue is not testable (Archibald et al., 2006a). Most workers have used the Nei–Li method when analyzing AFLP data, an approach that is probably conservative and has intuitive appeal because the method only takes into account the shared presence of fragments (Archibald et al., 2006b). Thus we present a consensus dendrogram generated with this method. Nodal support across this consensus neighbor-joining dendrogram was evaluated with bootstrapping (1000 replicates). Because inferences drawn from AFLP data sets can be sensitive to methodology (Meudt and Clarke, 2007), we also performed principal coordinate analysis to segregate groups with the marker data set using the program GENALEX version 6.1 (Peakall and Smouse, 2006). In addition, clustering of AFLP genotypes was investigated using the STRUCTURE program (Pritchard et al., 2000) with an assumed population size (*K*) of three, but without assigning the individuals to predefined groups. The analysis was based on 100 000 replications after 10 000 burnins using the admixture model and the assumption of independent allele frequencies among populations. The binary AFLP data set can be obtained by contacting the corresponding author.

RESULTS

Morphological and crossability analyses—The results of the morphological principal components analysis are plotted in Fig. 2. The first three components extracted from the data set

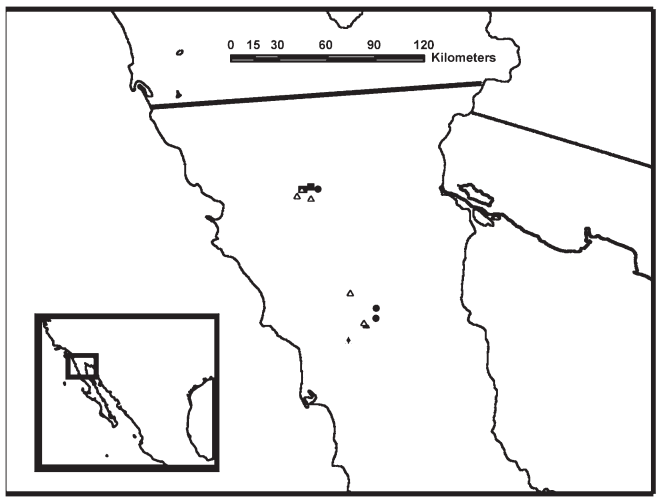

Fig. 1. Map of Giliopsis (*Ipomopsis*, Polemoniaceae) populations sampled. Filled circles: *I. effusa*. Filled squares: *I. guttata*. Open triangles: *I. tenuifolia*. The open triangle with a filled diamond represents the sympatric locality of *I. guttata* and *I. tenuifolia*. The northern group of localities is within the Sierra Juarez (SJ), the southern group within the Sierra San Pedro Martir (SSPM).

accounted for 86.1% of the variation. The three species are cleanly separated in morphospace. Thus, all three taxonomic species correspond to distinct morphological clusters. Given the genealogical results presented below, it is important to note that the SSPM individuals of *I. guttata* clearly group with their SJ conspecifics. In addition, while these SSPM plants are somewhat similar to experimental F1 hybrids between *I. guttata* (SJ) and *I. tenuifolia* with respect to corolla color, they do not overlap morphologically with experimental F1 plants when all the characters studied here are included (Fig. 2).

Fruit set and germination rates of seed obtained from controlled crosses are reported in Table 2. There was a marked reduction in fruit set in the two interspecific cross types relative to intraspecific crosses. Crosses between the annual, *I. effusa*, and *I. guttata* yielded some small fruits; however, only one seed from these fruits developed to maturity. This single seed germinated but died at the cotyledon stage. Thus, *I. effusa* is intrinsically isolated from the other two species. For the perennial taxa, when the data are pooled by cross type (intraspecific vs. interspecific), interspecific fruit set was significantly lower than intraspecific fruit set (Fisher's exact test, $P < 0.00001$). However, interspecific crossability was asymmetric vis-à-vis maternal parent: When *I. guttata* was the seed parent, fruit set was relatively high 82.95% (73/88) and not significantly different than fruit set in crosses among *I. guttata* individuals (Fisher's exact test, $P = 0.17356$). In contrast, when *I. tenuifolia* served as the maternal parent, fruit set was low, 9.27% (23/248). This asymmetry in fruit set is statistically significant (Fisher's exact test, $P < 0.00001$), and the difference in the pooled comparison can be attributed to the reduction in fruit set observed when *I. tenuifolia* is the seed parent. Given that *I. tenuifolia* pistil lengths were nearly twice as long as those of *I. guttata*, this asymmetry may be a function of the limited success of *I. guttata* pollen tubes in reaching *I. tenuifolia* ovules (e.g., Kiang and Hamrick, 1978). This asymmetric crossability may have important implications for patterns of gene flow between the species in nature.

Considering only the perennial taxa, on average, hybrid seeds were more likely to germinate than were seeds derived from intraspecific crosses (Table 2). When the data are pooled by cross type (intraspecific vs. interspecific), the difference in germination rate is statistically significant (Fisher's exact test, $P < 0.00002$). The germination rates reported here for the two perennial species were low, a pattern that is consistent with germination rates of field-collected seeds. Low germination success probably reflects a mismatch between the artificial conditions used and natural cues. Thus, while F1 hybrid seeds more readily germinated in artificial conditions, this observation should not be taken as strong evidence of F1 fitness heterosis. Overall, the crossability data suggest that there is potential for ongoing gene flow between the perennial taxa, a result that is in accord with the genetic patterns reported next.

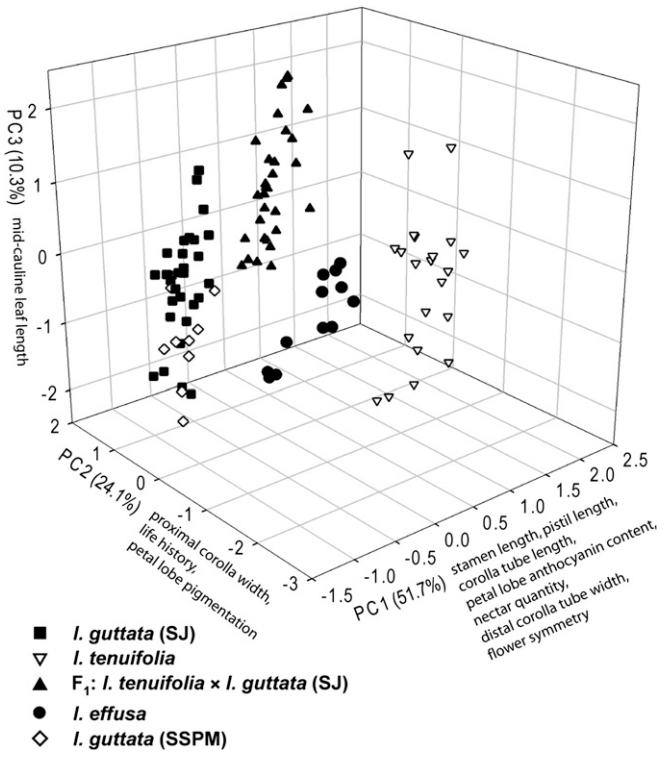

Fig. 2. Principal component analysis of morphometric and life history character variation of the three species of the Giliopsis group of *Ipomopsis*, and F1 hybrids (*I. guttata* × *I. tenuifolia*). The first three principal components along with character loadings are shown. SJ, Sierra Juarez; SSPM, Sierra San Pedro Martir.

AFLP analyses—For both Nei–Li and standard (mean character) distance measures, *I. guttata* by *I. tenuifolia* pairwise distances were always smaller than interspecific distances involving *I. effusa* individuals. This result suggests that *I. guttata* and *I. tenuifolia* comprise a lineage to the exclusion of *I. effusa* and is concordant with overall morphology. Based on this finding, *I. effusa* was used to root the neighbor-joining dendrogram for clarity of presentation (Fig. 3, left) and discussion. Indeed, neighbor-joining dendrograms reflect evolutionary relationships when shared character states outnumber ancestral or convergent states (Futuyma, 1998, p. 94; Tremetsberger et al., 2006). Here, we attempted to minimize homoplasy by analyzing only presence data (see Materials and Methods). In addition, rooting based on distance is justified if evolutionary rates are equal across lineages (Felsenstein, 1984), an assumption that is fundamental to evolutionary interpretation of distance analyses (Farris, 1972). Accurate inference from parsimony-based cladistic analyses is ultimately bound to this same rates assumption. Unfortunately, this assumption cannot be assessed empirically with the current data set.

In all bootstrap replicates, the *I. effusa* individuals grouped together (Fig. 3). The pattern of relationships among the perennial individuals was complex. Most notably, the SJ populations of *I. guttata* formed a lineage sister to a group containing the sampled SSPM population of *I. guttata* and all representatives of *I. tenuifolia*. The placement of the SSPM population of *I. guttata* within *I. tenuifolia* was reasonably well supported. Similarly, clustering analysis of the AFLP genotypes using STRUCTURE detected three distinct genotype groups corresponding to the three species, except that the genotypes of the SSPM population of *I. guttata* were indistinguishable from those of *I. tenuifolia* (Fig. 3; right). This result suggests that gene flow is limited and lineage sorting is nearly complete among the three groups, *I. effusa*, *I. guttata* (SJ), and *I. tenuifolia* + *I. guttata* (SSPM). In contrast, we found no evidence of barriers to gene flow between *I. tenuifolia* and *I. guttata* (SSPM), consistent with the PCO analysis (Fig. 4). Morphologically, however, individuals sampled from this SSPM population grouped with their *I. guttata* (SJ) conspecifics (Fig. 2). In both the morphological and AFLP ordinations, the SSPM population of *I. guttata* cannot be separated

TABLE 2. Crossability within and among species of the Giliopsis group (eff = *Ipomopsis effusa*, gut = *I. guttata*, tfl = *I. tenuifolia*). For the gut × tfl crosses, the maternal parent is written first. Data are pooled for the interspecific combinations involving eff. In addition, data are pooled by cross type across seed parents for both fruit set and germination rate. Number of seed parents: eff, $N = 7$; gut, $N = 10$; tfl, $N = 5$.

	Cross						
Crossability metric	eff × eff	gut × gut	tfl × tfl	gut × tfl	tfl × gut	eff × gut	eff × tfl
Fruits set	147	86	29	73	23	10	0
Flowers pollinated	174	115	42	88	248	100	12
Fruit set (%)	84.48	74.78	69.05	82.95	9.27	10.00	0
Seeds sown	80	100	40	100	19	1	0
Seeds germinated	63	14	5	35	9	1	0
Germination (%)	78.75	14.00	12.50	35.00	47.37	100.00	n/a

from its respective grouping by rotating the first three principal components. Taken together, these results indicate that neither of the morphologically coherent species, *I. tenuifolia* nor *I. guttata*, is monophyletic in the cladistic sense, when assessed genetically. Within *I. tenuifolia* + SSPM, there is little genetic structure that corresponds with geography, an outcome seen in other, similar studies (e.g., Archibald et al., 2004). This result, coupled with the limited sampling of the wide-ranging *I. tenuifolia*, demonstrates that detailed phylogeographic inference is not feasible here. In addition, the PCO results suggest that six individuals of *I. tenuifolia* (A1T3, A1T4, A4T1, A4T2, A5T2 and TP4) are more similar to the SSPM *I. guttata* cluster than are other *I. tenuifolia* individuals. However, only two of these individuals, A1T3 and A1T4, are drawn from the sympatric locality.

Finally, portions of the electropherograms for the only SJ sample of *I. effusa* (EFTH) from which DNA was obtained from dried material were poor. Consequently, presence/absence for these markers ($N = 597$ or 26%) was scored as unknown. Other workers have reported that DNA derived from herbarium material can be problematic for use with anonymous markers (Archibald et al., 2006b). Here, in the portions of the electropherograms that were included in the final data matrix, the quality of the genotypes for EFTH was subjectively equivalent to those from the other samples. Removal of this sample from the neighbor-joining analysis did not influence the topology in other parts of the dendrogram, and, when included, the sample grouped with conspecifics. Thus, there appears to be sufficient phylogenetic signal in the readable portion of the genotypes to warrant inclusion of this geographically important sample in all analyses.

DISCUSSION

Taxonomic and phylogenetic conclusions—The basic goal of this study was to assess species delimitation and relationships within the Giliopsis group as defined by Moran (1977). All three species are clearly distinct morphologically. The preponderance of vegetative, floral and life history characters, and crossability and genealogical data suggest that *I. guttata* and *I. tenuifolia* group to the exclusion of *I. effusa*. Thus, the phenotypic differences that are used to diagnose these two species represent a straightforward target of analysis for evaluating morphological speciation.

Discordance between morphology and molecules—Interestingly, the SSPM population of *I. guttata* groups with *I. tenuifolia* based on an extensive sampling of genomic variation, but with conspecifics based on a suite of morphological characters. The discordance is particularly surprising given that no morphologically intermediate individuals have been observed at the sympatric locality or, based on herbarium specimens, in other SSPM populations. This stark conflict between molecular and morphological markers in the absence of intermediates may be the result of strong disruptive selection that preserved typic floral morphologies after secondary contact and gene exchange. Other studies have documented the retention of morphological differentiation despite a lack of genetic differentiation (Streisfeld and Kohn, 2005; Currat et al., 2008), and a recent study describing a pattern similar to that seen here demonstrates that infrequent hybridization can lead to genomic homogenization while not blurring morphospecies boundaries (Barreto and McCartney, 2008). Given that our genealogical result is based on a genome-wide survey of variation, only genetic elements that determine floral morphology in *I. guttata* would be expected to cluster consistently to the exclusion of *I. tenuifolia* homologs, and vice versa, irrespective of population origin. That is, in this species pair, only genetic variation linked to factors underlying species diagnostic differences is expected to be reciprocally monophyletic.

The species problem and extensive gene flow—The results of this work highlight several key issues related to the species problem, a problem that has long fascinated evolutionists (Darwin, 1859; Dobzhansky, 1951; Mayr, 1957; Hey, 2001). In many plant groups, taxonomic species are not recovered as "monophyletic" when multiple populations are assessed with genetic data (e.g., Rieseberg and Brouillet, 1994; Tremetsberger et al., 2006; Ford et al., 2006). Similarly, many plant species are often not good genealogical species (sensu Baum and Shaw, 1995), an observation that is perhaps not surprising given the waiting time to complete reciprocal monophyly (Hudson and Coyne, 2002). This problem is particularly relevant to predicting the efficacy of efforts to assess plant species diversity with one or a few neutral markers (Chase et al., 2005; Lahaye et al., 2008). Even when taxa are completely reproductively isolated, the fixation of private alleles at neutral loci will lag behind the deterministic fixation of adaptive variants. Given our results in Giliopsis and other recent work showing that genomic differentiation between morphologically divergent lines can be minimal (e.g., Scotti-Saintagne et al., 2004; Streisfeld and Kohn, 2005; Yatabe et al., 2007; Barreto and McCartney, 2008), it appears that molecular taxonomic approaches will often fail to uncover genetic variation that correlates with ecologically relevant phenotypic variation used to diagnose species, particularly when closely related species co-occur or have diverged only recently.

The situation in Giliopsis is also pertinent to a long-standing debate over the importance of reproductive isolation to species differentiation. It is well known that many evolutionary biologists prefer to conceptualize species as reproductively independent lineages (Dobzhansky, 1951; Mayr, 1957; Coyne and Orr,

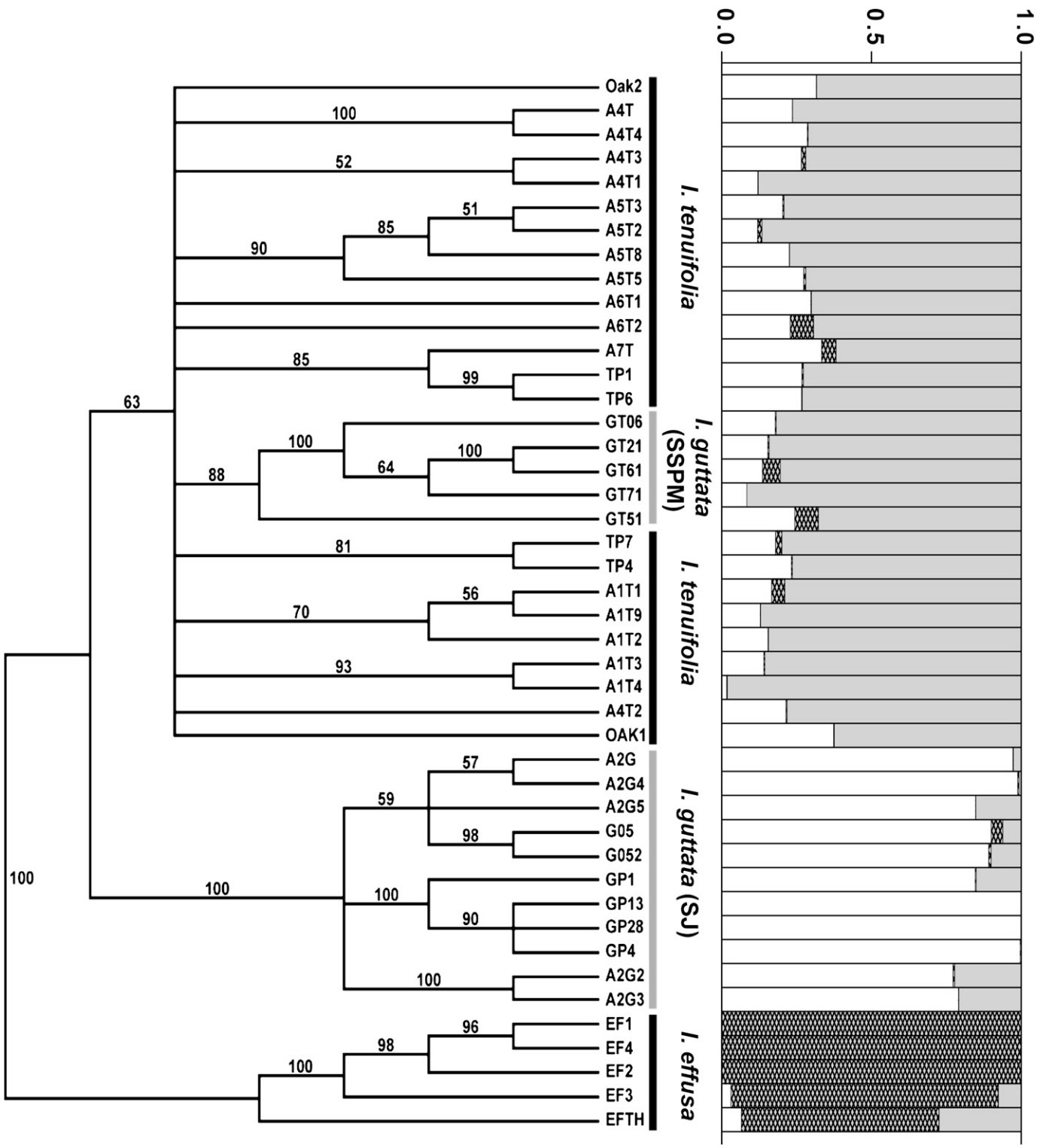

Fig. 3. AFLP-based inference of genetic relations among populations of the Giliopsis group. (Left) Dendrogram of Nei–Li distances based on 2333 AFLP markers for 44 individuals of the Giliopsis group of *Ipomopsis*. Numbers indicate bootstrap support for the nodes which they subtend. (Right) Results of a STRUCTURE analysis, constrained to three populations, showing the proportions of alleles derived from the three populations for each individual. SJ, Sierra Juarez; SSPM, Sierra San Pedro Martir.

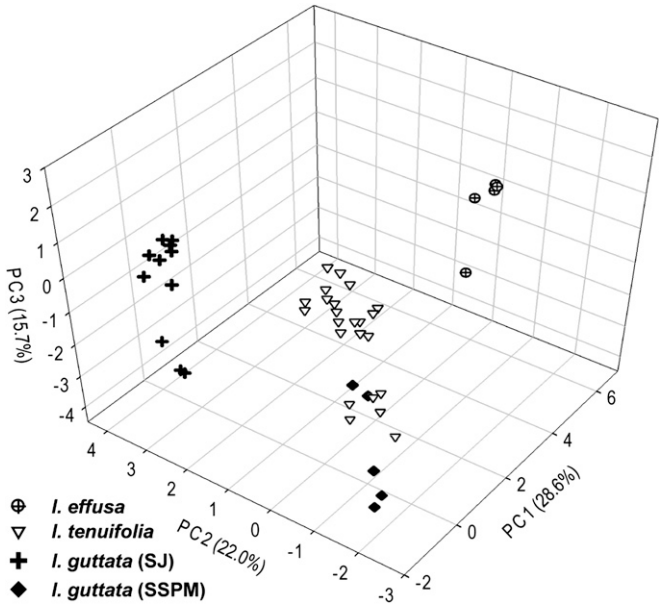

Fig. 4. Principal coordinate analysis of the AFLP data set (2333 markers) derived from 44 individuals representing the three species of the Giliopsis group of *Ipomopsis*. The first three principal coordinates and the proportion of the variance they explain are shown. SJ, Sierra Juarez; SSPM, Sierra San Pedro Martir.

2004), and such lineages are called biological species. Botanists have argued that in many plant groups, divergent adaptation/speciation is not dependent on reproductive isolation (e.g., Grant, 1957). While intrinsic reproductive isolation is positively correlated with the morphological discreteness of plant species (Rieseberg et al., 2006), this association does not mean that reproductive isolation is necessary. In Giliopsis, the perennial taxa are clearly good morphological species, but the data presented here make it clear that they are not good biological species. With respect to premating isolation, observations of pollinators visiting an artificial hybrid population demonstrate that hummingbirds visit *I. tenuifolia* plants at twice the rate that they visit *I. guttata* plants, but they also visit the latter and *I. guttata*-like hybrids with substantial frequency (T. Wood, unpublished data). In contrast, insect visits to *I. tenuifolia* are rare (T. Wood, unpublished data). However, these observations were obtained from an artificial population in which floral variation was distributed unimodally between the F1 and *I. guttata* forms (pure parents, F1s, and backcross hybrids to *I. guttata*), and pollinator behavior in this array may not be fairly generalized to the natural sympatric population studied here, where individual floral characters are distributed bimodally. Indeed, hummingbirds (*Calyptae costae*) at the sympatric locality in SSPM did not visit *I. guttata* plants, but only 19 foraging bouts have been observed at this site. The occurrence of hybrids at contact zones of certain species of the *Ipomopsis aggregata* complex demonstrates that pollinator fidelity is not strong enough to prevent interspecific gene exchange in this group (Grant and Wilken, 1988).

The SSPM population of *I. guttata* is represented in the AFLP and morphological analyses by plants grown from seed collected from wild plants. While habitat specificity may lead to genotype by environment isolation, the range of *I. guttata* is nested within that of *I. tenuifolia*. The sympatric occurrence of the two species indicates that habitat-dependent postzygotic isolation, if it exists, is incomplete. Considering the entire data set presented here, the most plausible historical hypothesis is that hummingbirds have transported genes to *I. guttata* plants, but selection against morphologically intermediate segregants is strong. Similar patterns have been seen in other groups, e.g., fish (Barreto and McCartney, 2008) and *Mimulus auraniaicus* (Streisfeld and Kohn, 2005). In both of these cases, neutral markers were homogenous despite color differentiation, which is known to often have a simple, sometimes single-locus, genetic basis. In the case of Giliopsis, however, differentiation in a suite of traits is maintained despite genetic homogenization.

So what is the best way to conceptualize species and speciation in Giliopsis? An insightful approach was outlined by Mallet (1995), who argued that speciation can only be distinguished from adaptation when divergent lineages are in contact, and in these cases, speciation boils down to a balance between disruptive selection on and gene flow at genetic loci that underlie diagnostic characters. This view effectively conciliates the isolation vs. morphologic concepts of species, because strong selection against intermediate states of diagnostic characters is equivalent to reproductive isolation. As we look closer and closer at the partitioning of genetic variation across congeneric plant species, we often find very little genomic differentiation (Yatabe et al., 2007; Minder and Widmer, 2008; Stadler et al., 2008). In these instances, regions of the genome that are differentiated are likely distinct due to divergent selection. These regions are the stuff of speciation, as they underlie Darwin's (1859) "divergence of character." In understanding the speciation of the perennial Giliopsis, the ultimate challenge, from an evolutionary genetic perspective, is to identify species-specific genetic variation that is causal to the character variation used by Gray (1876) to describe them.

Synopsis of Giliopsis—The three species of the Giliopsis group are good morphological species, and the morphological characters used to separate them appear to indicate ecological (pollinator) differences. The most recent speciation event in the group is that which separates the two perennial taxa, *I. guttata* and *I. tenuifolia*. The manner in which genetic variation is partitioned across this morphological species boundary adds to a growing body of data (Yatabe et al., 2007; Barreto and McCartney, 2008; Minder and Widmer, 2008; Stadler et al., 2008) that reveal that patterns of variation in genetic markers frequently do not corroborate morphology-based taxonomies. In general, such data suggest that attempts to use one or a few molecular markers to assess species diversity will often be misleading, especially when sampled regions contain closely related taxa. More positively, the fact that species-specific genetic variation is often limited to relatively small regions of the genome presents promising opportunities for identifying the relevant genomic components of character divergence (Lexer and Widmer, 2008). Because of their high interfertility and retention of morphological integrity in sympatry, *I. guttata* and *I. tenuifolia* provide a particularly promising model for identifying the genetic determinants of speciation.

LITERATURE CITED

ARCHIBALD, J. K., D. J. CRAWFORD, A. SANTOS-GUERRA, AND M. E. MORT. 2006b. The utility of automated analysis of inter-simple sequence repeat (ISSR) loci for resolving relationships in the Canary Island

species of *Tolpis* (Asteraceae). *American Journal of Botany* 93: 1154–1162.

ARCHIBALD, J. K., M. E. MORT, D. J. CRAWFORD, AND A. SANTOS-GUERRA. 2006a. Evolutionary relationships within recently radiated taxa: Comments on methodology and analysis of inter-simple sequence repeat data and other hypervariable, dominant markers. *Taxon* 55: 747–756.

ARCHIBALD, J. K., A. D. WOLFE, AND S. D. JOHNSON. 2004. Hybridization and gene flow between a day- and night-flowering species of *Zaluzianskya* (Scrophulariaceae S.S., Tribe *Manuleeae*). *American Journal of Botany* 91: 1333–1344.

AVISE, J. C., AND K. WOLLENBERG. 1997. Phylogenetics and the origin of species. *Proceedings of the National Academy of Sciences, USA* 94: 7748–7755.

BARRETO, F. S., AND M. A. MCCARTNEY. 2008. Extraordinary AFLP fingerprint similarity despite strong assortative mating between reef fish color morphospecies. *Evolution* 62: 226–233.

BAUM, D. A., AND K. L. SHAW. 1995. Genealogical perspectives on the species problem. *In* P. C. Hoch and A. C. Stephenson [eds.], Experimental and molecular approaches to plant biosystematics, 289–303. Missouri Botanical Garden Press, St. Louis, Missouri, USA.

CAMPBELL, D. R. 2004. Natural selection in *Ipomopsis* hybrid zones: Implications for ecological speciation. *New Phytologist* 161: 83–90.

CAMPBELL, D. R., N. M. WASER, M. V. PRICE, E. A. LYNCH, AND R. J. MITCHELL. 1991. Components of phenotypic selection: Pollen export and lower corolla width in *Ipomopsis aggregata. Evolution* 45: 1458–1467.

CHASE, M. W., N. SALAMIN, M. WILKINSON, J. M. DUNWELL, R. P. KESANAKURTHI, N. HAIDAR, AND V. SAVOLAINEN. 2005. Land plants and DNA barcodes: Short-term and long-term goals. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* 360: 1889–1895.

COYNE, J. A., AND H. A. ORR. 2004. Speciation. Sinauer, Sunderland, Massachusetts, USA.

CRONQUIST, A. 1988. The evolution and classifcation of flowering plants. New York Botanical Garden, New York, New York, USA.

CURRAT, M., M. RUEDI, R. J. PETIT, AND L. EXCOFFIER. 2008. The hidden side of invasions: Massive introgression by local genes. *Evolution* 62: 1908–1920.

DARWIN, C. 1859. On the origin of species by means of natural selection or the preservation of favored races in the struggle for life. J. Murray, London, UK.

DOBZHANSKY, T. 1951. Genetics and the origin of species. Columbia University Press, New York, New York, USA.

DOYLE, J. J. 1992. Gene trees and species trees—Molecular systematics as one-character taxonomy. *Systematic Botany* 17: 144–163.

FARRIS, J. S. 1972. Estimating phylogenetic trees from distance matrices. *American Naturalist* 106: 645–668.

FELSENSTEIN, J. 1984. Distance methods for inferring phylogenies—A justification. *Evolution* 38: 16–24.

FORD, V. S., J. LEE, B. G. BALDWIN, AND L. D. GOTTLIEB. 2006. Species divergence and relationships in *Stephanomeria* (Compositae): *PgiC* phylogeny compared to prior biosystematic studies. *American Journal of Botany* 93: 480–490.

FUTUYMA, D. 1998. Evolutionary biology. Sinauer, Sunderland, Massachusetts, USA.

GRANT, V. 1949. Pollination systems as isolating mechanisms in angiosperms. *Evolution* 3: 82–97.

GRANT, V. 1956. A synopsis of *Ipomopsis. Aliso* 3: 351–362.

GRANT, V. 1957. The plant species in theory and practice. *In* E. Mayr [ed.], The species problem. American Association for the Advancement of Science, Washington, D.C., USA.

GRANT, V., AND K. GRANT. 1965. Flower pollination in the phlox family. Columbia University Press, New York, New York, USA.

GRANT, V., AND D. H. WILKEN. 1988. Natural hybridization between *Ipomopsis aggregata* and *Ipomopsis tenuituba* (Polemoniaceae). *Botanical Gazette (Chicago, Ill.)* 149: 213–221.

GRAY, A. 1876. Miscellaneous botanical contributions. *Proceedings of the American Academy of Arts and Sciences* 11: 71–104.

GRAY, A. 1885. Contributions to the botany of North America. *Proceedings of the American Academy of Arts and Sciences* 20: 257–310.

HENRICKSON, J. 1987. New species, combinations, and notes in *Ipomopsis. Aliso* 11: 589–598.

HEY, J. 2001. Genes, categories, and species. Oxford University Press, Oxford, UK.

HUDSON, R. R., AND J. A. COYNE. 2002. Mathematical consequences of the genealogical species concept. *Evolution* 56: 1557–1565.

ISAAC, N. J. B., J. MALLET, AND G. M. MACE. 2004. Taxonomic inflation: Its influence on macroecology and conservation. *Trends in Ecology & Evolution* 19: 464–469.

KIANG, Y. T., AND J. L. HAMRICK. 1978. Reproductive isolation in the *Mimulus guttatus–M. nasutus* complex. *American Midland Naturalist* 100: 269–276.

KIM, S. C., AND L. H. RIESEBERG. 1999. Genetic architecture of species differences in annual sunflower: Implications for adaptive trait introgression. *Genetics* 153: 965–977.

KOOPMAN, W. J. M. 2005. Phylogenetic signal in AFLP data sets. *Systematic Biology* 54: 197–217.

LAHAYE, R., M. VAN DER BANK, D. BOGARIN, J. WARNER, F. PUPULIN, G. GIGOT, O. MAURIN, ET AL. 2008. DNA barcoding the floras of biodiversity hotspots. *Proceedings of the National Academy of Sciences, USA* 105: 2923–2928.

LEXER, C., AND A. WIDMER. 2008. The genic view of plant speciation: Recent progress and emerging questions. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* 363: 3023–3036.

MALLET, J. 1995. A species definition for the Modern Synthesis. *Trends in Ecology & Evolution* 10: 294–299.

MAYR, E. 1957. The species problem. American Association for the Advancement of Science, Washington, D.C., USA.

MEUDT, H. M., AND A. C. CLARKE. 2007. Almost forgotten or latest practice? AFLP applications, analyses and advances. *Trends in Plant Science* 12: 106–117.

MICHENER, C. D. 1970. Diverse approaches to systematics. *In* T. Dobzhansky, M. K. Hecht, and W. C. Steere [eds.] Evolutionary biology, vol. 4. Appleton-Century-Crofts, New York, New York, USA.

MINDER, A. M., AND A. WIDMER. 2008. A population genomic analysis of species boundaries: Neutral processes, adaptive divergence and introgression between two hybridizing plant species. *Molecular Ecology* 17: 1552–1563.

MINNICH, R. A., AND E. FRANCO-VIZCAINO. 1998. Land of chamise and pines: historical accounts and current status of vegetation in northern Baja California. *University of California Publications in Botany* 80: 1–166.

MITCHELL, R. J., AND N. M. WASER. 1992. Adaptive significance of *Ipomopsis aggregata* nectar production: Pollination success of single flowers. *Ecology* 73: 633–638.

MORAN, R. 1977. New or renovated Polemoniaceae from Baja California, Mexico (*Ipomopsis*, *Linanthus*, *Navarretia*). *Madroño* 24: 141–159.

MUELLER, U., AND L. WOLFENBARGER. 1999. AFLP genotyping and fingerprinting. *Trends in Ecology & Evolution* 14: 389–394.

O'HANLON, P. C., AND R. PEAKALL. 2000. A simple method for the detection of size homoplasy among amplified fragment length polymorphism fragments. *Molecular Ecology* 9: 815–816.

PEAKALL, R., AND P. E. SMOUSE. 2006. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.

POLLARD, D. A., V. N. IYER, A. M. MOSES, AND M. B. EISEN. 2006. Widespread discordance of gene trees with species tree in *Drosophila*: Evidence for incomplete lineage sorting. *Public Library of Science Genetics* 2: 1634–1647.

PRITCHARD, J. K., M. STEPHENS, AND P. J. DONNELLY. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945–959.

RIESEBERG, L. H., AND L. BROUILLET. 1994. Are many plant species paraphyletic? *Taxon* 43: 21–32.

RIESEBERG, L. H., T. E. WOOD, AND E. J. BAACK. 2006. The nature of plant species. *Nature* 440: 524–527.

ROKAS, A., B. L. WILLIAMS, N. KING, AND S. B. CARROLL. 2003. Genome-scale approaches to resolving incongruence in molecular phylogenies. *Nature* 425: 798–804.

ROKAS, A., AND S. B. CARROLL. 2006. Bushes in the tree of life. *Public Library of Science Biology* 4: 1899–1904.

SAS Institute. 1989–2002. JMP, version 5. SAS Institute, Cary, North Carolina, USA.

SCOTTI-SAINTAGNE, C., S. MARIETTE, I. PORTH, P. G. GOICOECHEA, T. BARRENECHE, K. BÓDENÈS, K. BURG, AND A. KREMER. 2004. Genome scanning for interspecific differentiation between two closely related oak species [*Quercus robur* L. and *Q. petraea* (Matt.) Lieble.]. *Genetics* 168: 1615–1626.

STADLER, T., U. ARUNYAWAT, AND W. STEPHAN. 2008. Population genetics of speciation in two closely related wild tomatoes (*Solanum* section *Lycopersion*). *Genetics* 178: 339–350.

STREISFELD, M. A., AND J. R. KOHN. 2005. Contrasting patterns of floral and molecular variation across a cline in *Mimulus auranticus*. *Evolution* 59: 2548–2559.

SWOFFORD, D. L. 2002. PAUP*: Phylogenetic analyses using parsimony (*and other methods), version 40b10. Sinauer, Sunderland, Massachusetts, USA.

SYSTAT SOFTWARE. 2004. SigmaPlot 9.0 for Windows. Systat Software, Point Richmond, California, USA.

TREMETSBERGER, K., T. F. STUESSY, G. KADLEC, E. URTUBEY, C. M. BAEZA, S. G. BECK, H. A. VALDEBENITO, ET AL. 2006. AFLP phylogeny of South American species of *Hypochaeris* (Asteraceae, Lactuceae). *Systematic Botany* 31: 610–626.

VOS, P., R. HOGERS, M. BLEEKER, M. REIJANS, T. VANDELEE, M. HORNES, A. FRIJTERS, ET AL. 1995. AFLP—A new technique for DNA-fingerprinting. *Nucleic Acids Research* 23: 4407–4414.

WILKEN, D. H. 1982. A simple method for estimating anthocyanin concentrations in tissue extracts. *Phytochemical Bulletin* 15: 7–13.

YATABE, Y., N. C. KANE, C. SCOTTI-SAINTAGNE, AND L. H. RIESEBERG. 2007. Rampant gene exchange across a strong reproductive barrier between the annual sunflowers, *Helianthus annuus* and *H. petiolaris*. *Genetics* 1 5: 1883–1893.

APPENDIX 1. Populations sampled and associated voucher specimens. Voucher specimens are at Deam Herbarium, Indiana University = IND and Rancho Santa Ana Botanic Garden = RSA.

Taxon—Population codes, *Voucher specimen*; Herbarium = IND unless otherwise noted.

Ipomopsis effusa (A. Gray) Moran—EF1, EF2, *Wood 11*; EF3, EF4, *Wood 12*; EFTH, *RF Thorne 60529*, RSA. ***Ipomopsis guttata*** (A. Gray) Moran—GT06, GT21, GT51, GT61, GT71, Wood 2; A2G, A2G2, A2G3, A2G4, A2G5, Wood 16; GP1, GP4, GP13, GP28, Wood 15; G05, G052, Wood 22. ***Ipomopsis tenuifolia*** (A. Gray) V.E. Grant—A1T1, A1T2, A1T3, A1T4, A1T9, Wood 1; TP1, TP4, TP6, TP7, Wood 9; Oak 1, Oak2, Wood 3; A4T, A4T1, A4T2, A4T3, A4T4, Wood 4; A5T2, A5T3, A5T5, A5T8, Wood 5; A6T1, A6T2, Wood 6; A7T, Wood 7.