Evolution, 57(6), 2003, pp. 1397–1410

AFLP PHYLOGENY OF *MIMULUS* SECTION *ERYTHRANTHE* AND THE EVOLUTION OF HUMMINGBIRD POLLINATION

PAUL M. BEARDSLEY,[1] ALAN YEN, AND RICHARD G. OLMSTEAD
University of Washington, Department of Botany, Box 355325, Seattle, Washington 98115
[1]*E-mail: pbeard@u.washington.edu*

Abstract.—Species in *Mimulus* section *Erythranthe* (monkeyflowers) have become model systems for the study of the genetic basis of ecological adaptations. In this study, we pursued two goals. First, we reconstructed the phylogeny of species in *Erythranthe* using both DNA sequences from the ribosomal DNA ITS and ETS and AFLPs. Data from rDNA sequences support the monophyly of the section, including *M. parishii*, but provide little support for relationships within it. Analyses using AFLP data resulted in a well-supported hypothesis of relationships among all *Erythranthe* species. Our second goal was to reconstruct ancestral pollination syndromes and ancestral states of individual characters associated with hummingbird-pollinated flowers. Both parsimony and likelihood approaches indicate that hummingbird pollination evolved twice in *Erythranthe* from insect-pollinated ancestors. Our reconstruction of individual characters indicates that corolla color and some aspects of corolla shape change states at the same point on the phylogenetic tree as the switch to hummingbird pollination; however, a switch to secretion of high amounts of nectar does not. Floral trait transformation may have been more punctuational than gradual.

Key words.—Amplified fragment-length polymorphisms, *Erythranthe*, external transcribed spacer, hummingbird-pollination, internal transcribed spacer, *Mimulus*.

Received February 19, 2002. Accepted January 2, 2003.

Well-resolved species-level phylogenies and estimates of ancestral states are necessary to interpret and understand the evolution of interesting ecological adaptations between species. Ancestral pollination syndromes and character states in hummingbird-pollinated plants have received particular attention from plant evolutionary biologists. Stebbins (1989) estimated that 108 of 129 hummingbird-pollinated taxa in the western United States have sister taxa that are insect-pollinated with hummingbird pollination being the derived state. In a review of attempts to use phylogenies to determine the direction of shifts in pollination syndromes, Weller and Sakai (1999) report three studies in which the evolution of hummingbird pollination has been investigated (McDade 1992; Bruneau 1997; Hodges 1997). McDade (1992) found that long, decurved corollas associated with pollination by specialist hermit hummingbirds are ancestral to short corollas associated with pollination by generalist hummingbirds. In the *Aphelandra pulcherrima* complex, short corollas were hypothesized to have arisen twice. Hummingbird pollination in *Erythrina* was estimated by Bruneau (1997) to have arisen at least four times from ancestral passerine pollination. *Aquilegia* species that are pollinated by hummingbirds were hypothesized by Hodges (1997) to be derived from taxa with less-specialized, open, radiate corollas. O'Kane and Schaal (1998) used a molecular phylogeny to infer that hummingbird pollination in *Lopezia* sect. *Jehlia* was secondarily regained after a switch to fly pollination. Givnish et al. (2000) also used molecular data to infer the relatively recent evolution of hummingbird pollination from bee-pollinated ancestors in Rapateaceae in the western Guyana Shield. Hummingbird pollination was hypothesized to have arisen three times independently from bee-pollinated ancestors in tribe Antirrhineae (Veronicaceae, sensu Olmstead et al. 2001; Ghebrehiwet et al. 2000). Similarly, in our broad-scale analyses of phylogenetic relationships within *Mimulus* and closely related genera, we demonstrated that within the newly redefined Phrymaceae, hummingbird pollination has arisen independently at least three times from insect-pollinated ancestors (Beardsley and Olmstead 2002).

Both Mullerian and Batesian mimicry have been suggested as mechanisms by which hummingbird pollination can evolve in western North America (Grant 1966; Brown and Kodrick-Brown 1979; Bleiweiss 2001). Most hummingbird species in western North America are migratory, thus requiring them to be able to quickly identify food sources in new areas (Grant 1966). According to this hypothesis, hummingbirds learn to associate floral color (red) and shape with nectar rewards. Convergence in floral design and rewards thus increases hummingbird pollination through Mullerian mimicry. Batesian mimicry occurs if rare populations or species possess convergent floral displays without offering nectar rewards (Brown and Kodrick-Brown 1979).

Species in *Mimulus* section *Erythranthe* (monkeyflowers) have become one of the most thoroughly studied model systems for the study of ecological adaptations (Hiesey et al. 1971), including adaptations to different pollinators (Sutherland and Vickery 1993; Vickery 1995, Bradshaw et al. 1995, 1998). Five of six species traditionally placed in *Erythranthe* are hummingbird-pollinated (*M. cardinalis*, *M. eastwoodiae*, *M. verbenaceus*, *M. nelsonii*, and *M. rupestris*) and one is bee-pollinated (*M. lewisii*; Sutherland and Vickery 1993). *Erythranthe* played an important role in Vickery's analysis of evolution in species complexes (Vickery 1978) and his studies on color polymorphisms and their effect on pollinator visitation (Sutherland and Vickery 1993) and their potential role in reproductive isolation (Vickery 1995). Studies on the genetic basis for floral differences between *M. lewisii* and *M. cardinalis* (Bradshaw et al. 1995, 1998; Schemske and Bradshaw 1999) strongly suggest that quantitative trait loci (QTLs) of large effect have been important for the evolution of traits associated with pollinator preference and, thus, reproductive isolation between these taxa. These studies have also added insight into the genetics of mimicry (Bleiweiss 2001). Further insight into the evolution of evolution-

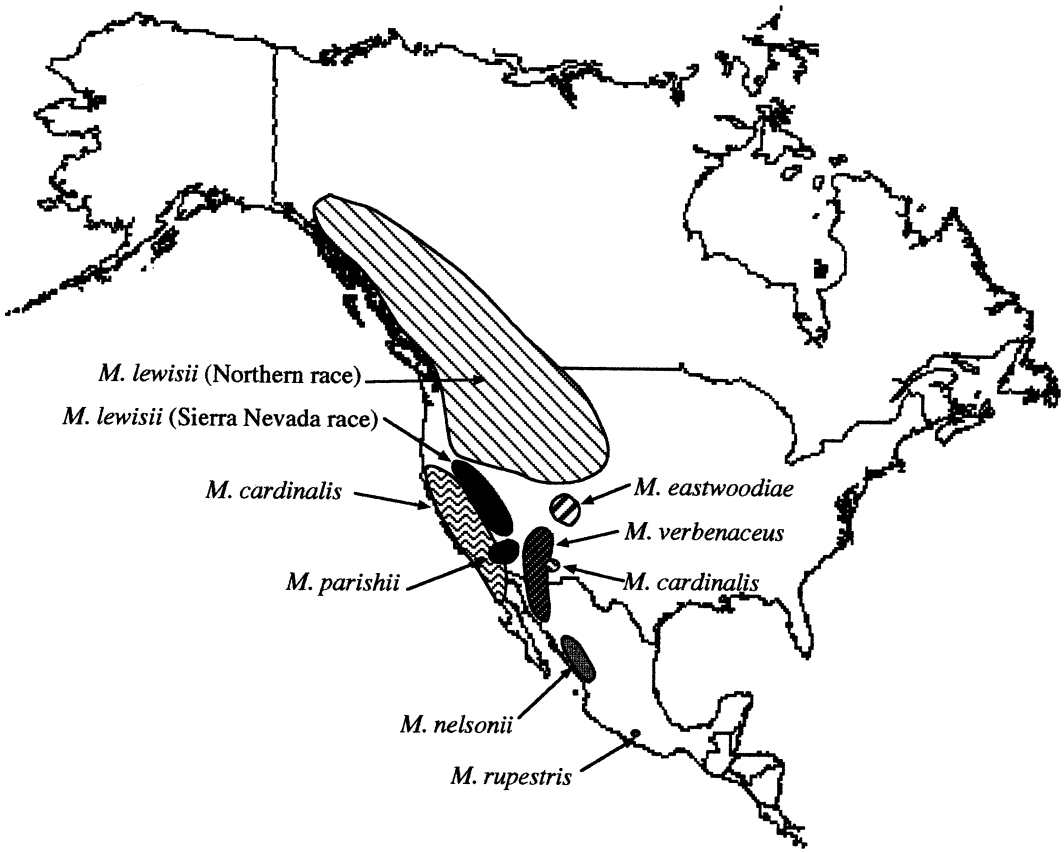

FIG. 1. Distribution map of taxa identified as belonging to a monophyletic *Erythranthe*.

ary processes in *Erythranthe* has been limited, however, because no well-resolved phylogeny exists for members of the section, although they have been the subject of many systematic studies (Grant 1924; Hiesey et al. 1971; Vickery and Wullstein 1987).

Thus, the first goal of this study was to estimate species-level phylogenies in *Mimulus* section *Erythranthe* using two methods; sequence data from the nuclear ribosomal (nr) DNA internal and external transcribed spacer (ITS and ETS, respectively; Baldwin et al. 1995; Baldwin and Markos 1998) regions, and data from amplified fragment length polymorphisms (AFLPs; Vos et al. 1995). Recent studies of ITS and ETS sequences within *Mimulus* (Beardsley and Olmstead 2001) indicate that species in *Erythranthe* are part of a recent radiation, a fact that has made phylogenetic reconstruction solely with ITS and/or ETS more challenging in other groups (*Aquilegia* [Hodges 1997]; *Bidens* [Ganders et al. 2000]). Polymorphic DNA-based markers, such as AFLPs, have been useful in plant phylogenetic studies (Wolfe and Liston 1998; Caicedo et al. 1999; Xu and Sun 2001; Zhang et al. 2001) when DNA sequence variation is limited. The second goal of this analysis was to reconstruct the evolution of pollination syndromes in *Erythranthe* and to infer the ancestral states of a suite of traits associated with hummingbird pollination. Ancestral character states were optimized using both maximum parsimony (Maddison and Maddison 1992) and maximum-likelihood (Pagel 1994). Maximum likelihood methods have the advantage of allowing uncertainty to be quantified (Cunningham et al. 1998). Reconstructing the sequence of character evolution within *Erythranthe* provides an empirical case study of the hypothesis that hummingbird-pollinated taxa are derived from insect-pollinated ancestors in western North America (Grant and Grant 1968) and an important context for understanding the genetic changes between *M. lewisii* and *M. cardinalis* (Bradshaw et al. 1995, 1998).

MATERIALS AND METHODS

Sampling

Sampling in the ITS/ETS study was designed to examine the monophyly of section *Erythranthe* and to determine appropriate outgroups within *Mimulus*. Included in this analysis are the six species in the currently described section *Erythranthe*, plus 13 other *Mimulus* species to serve as outgroups and to identify the sister species or clade for *Erythranthe* (Table 1). Multiple individuals were sampled for most of the species in *Erythranthe* throughout their respective ranges (Fig. 1). Most of the material for individuals in *Erythranthe* came from herbarium specimens. Results from molecular systematic analyses currently underway (P. Beardsley, unpubl. data) of nearly every species of *Mimulus* indicate that our sample contains all the species that are closely related to *Erythranthe*.

Due to the variability of AFLP markers and the increasing probability of detected fragments being homoplastic with increasing genetic distance between taxa, only comparisons

among closely related taxa are appropriate (Mueller and Wolfenbarger 1999). Thus, only taxa in *Erythranthe* and in its sister clade were sampled in our AFLP study. Individual accessions sampled for the AFLP study within *Erythranthe* differ from those in the ITS/ETS study because fresh or silica-dried material is necessary for generating AFLPs. When conducting analyses with highly polymorphic markers, it is vital to obtain an appropriate frame of reference by sufficiently sampling intraspecific variation (Avise 1994). As a result, many populations for each taxon were collected, with the exception of *M. nelsonii* and the rare *M. rupestris*, which were provided by Bob Vickery (Table 1). An attempt was made to sample across the geographic range of each taxon.

ITS/ETS Analysis

The modified cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) was used to extract total genomic DNA, which was further purified using Qiaquick spin-columns (Qiagen, Valencia, CA). The entire ITS region was amplified using its4 and its5 primers (Baldwin 1992). To amplify a portion of the ETS, we used the 3′ 18S-IGS primer of Baldwin and Markos (1998). The 5′ primer, ETS-B (5′- ATAGAGCGCGTGAGTGGTG-3′), was designed using *Mimulus* sequences as a reference. The polymerase chain reaction (PCR) conditions were: 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Polymerase chain reaction products were purified using Qiaquick spin-columns according to the manufacturer's protocol. Sequences were generated on an ABI 377 (Applied Biosystems, Foster City, CA) for both strands of the PCR product. ITS was sequenced using the external PCR primers, its4 and its5, and the two internal primers, its2 and its3. ETS was sequenced using the 18S-E primer of Baldwin and Markos (1998), which is slightly internal to 18S-IGS primer, and the ETS-B primer. Electropherograms for each region were compiled and compared using the program Sequencher version 3.0 (Gene Codes Corporation, Ann Arbor, MI), from which a consensus sequence was generated. Consensus sequences for ITS and ETS were aligned manually.

Sequence data was analyzed using PAUP* 4.0b3a (Swofford 1998). The incongruence length difference (ILD) test (Farris et al. 1994, as implemented in PAUP*) was used to assess potential conflicts between the phylogenetic signal from ITS and ETS. In this test, 500 replicates were analyzed with a heuristic search, each with 10 random sequence addition replicates with MULTREES and TBR branch swapping, but limited to 1000 trees. Parsimony searches were conducted using heuristic searches with 10,000 random sequence addition replicates, TBR swapping and MULTREES to find multiple tree islands, if present (Maddison 1991). Identical sequences were excluded from the analysis. Gaps were scored as missing data. Support for individual branches was estimated using the bootstrap (Felsenstein 1985), which was calculated using 1000 replicate heuristic searches with MULTREES and TBR branch swapping, limited to 1000 trees per replicate.

AFLP Analysis

The modified CTAB method of Doyle and Doyle (1987) was used to extract total genomic DNA, which was further purified using Qiaquick spin-columns. DNA samples were quantified with a spectrophotometer. Two sampling strategies were employed. First, AFLP profiles were generated for every individual in the study. Second, after confirming that named species were monophyletic, DNA from one to three individuals, selected to represent the range of diversity present in the analysis of individuals, was bulked and AFLPs were separately developed and screened for the seven species in *Erythranthe* and one outgroup (Wolfe and Liston 1998).

Procedures for AFLPs were as described by Vos et al. (1995) with some modifications. Digestion and ligation: 400–500 ng DNA of each sample was digested and ligated with 5 U of *Eco*RI and *Mse*I in a reaction mixture that contained 5 μL 10X RL buffer (100 mM tris-acetate pH 7.6, 100 mM Mg-acetate, 500 mM K-acetate, 50 mM dithiothreitol), 5 pmol *Eco*RI adapter, 50 pmol *Mse*I adapter, 1 μL 10 mM ATP, 1 U T4-DNA ligase, and water to a final volume of 50 μL. Adapter sequences are the same as those described by Xu et al. (2000). After digestion and ligation, products were diluted 1:4 with water.

In the first round of PCR (pre-selective amplification), each reaction contained 1.2 μL Promega (Madison, WI) 10X reaction buffer, 0.8 μL MgCl2 (25mM), 0.4 μL dNTPs (10 mM), 14.32 μL water, 0.08 μL Promega Taq Polymerase, 0.6 μL *Mse*I + C primer (50 ng/μL), 0.6 μL *Eco*RI + A primer (50 ng/μL), and 2.0 μL of the diluted digestion/ligation product. The PCR conditions were: 94°C for 30 sec, 60°C for 60 sec, and 72°C for 60 sec, for 28 cycles. The PCR product was diluted 1:20 with water. Samples used in the bulked DNA study were run individually through the preselective amplification step. Subsequently, 10 μL of the diluted preselective amplification from each of three individuals was combined. The combined product was used for the selective amplification step. Specific control amplifications were performed to ensure that this dilution of the preselective amplification did not influence the number or size of bands recovered in the bulked DNA analysis.

In the second round of PCR (selective amplification), each reaction contained 2.0 μL Promega 10X Reaction Buffer, 1.6 μL MgCl2 (25 mM), 0.4 μL dNTPs (10 mM), 11.275 μL H2O, 0.1 μL Promega Taq Polymerase, 0.625 μL *Mse*I + XXX primer (50 ng/μL), 1.0 μL *Eco*RI + XXX (+ dye) primer (1 ng/μL), 3.0 μL diluted preselective product. The PCR conditions were: 94°C for 2 min, 65°C for 30 sec, 72°C for 2 min, 1 cycle; 94°C for 1 sec, 64°C for 30 sec (−1.0°C/cycle), 72°C for 2 min, 8 cycles; 94°C for 1 sec, 56°C for 30 sec, 72°C for 2 min, 23 cycles. All samples were processed in random order and subsamples were re-run for internal control.

Samples were prepared for analysis on an ABI 377 by combining 0.75 μL of selective amplification product with 1.5 μL of loading buffer. Loading buffer is made using 180 μL formamide, 50 μL GeneScan 500-Tamara size standard, and 70 μL blue loading dye (comes with standard). Samples were run on a 4.0% denaturing polyacrylamide gel. Raw data were collected using ABI GeneScan software (Applied Biosystems). Amplification products were sized in reference to the size standard using the local Southern method, which allowed samples from different gels to be aligned accurately. Aligned data were imported into Genographer (ver. 1.4, Mon-

TABLE 1. Taxa sampled in this study, including their population designation. Also included are the location where the sample was collected, and voucher and herbarium information. WTU, University of Washington; UC, University of California; UTC, Utah State University; ASU, Arizona State University; JEPS, Jepson Herbarium; ARIZ, University of Arizona; CDA, California Department of Food and Agriculture; OSC, Oregon State University. Note that the individuals sampled in the ITS/ETS study are different than those sampled in the AFLP study except those noted with an asterisk.

ITS/ETS study			AFLP study		
Species/sample	Voucher/herbarium	Location	State/sample	Species	Voucher
Mimulus cardinalis 1	Alexander and Kellogg 5583 WTU	CA	OR 01	*M. cardinalis*	SS 98-75 WTU
M. cardinalis 2	Morefield 4023 UC	CA	CA 02	*M. cardinalis*	PB 98-050 WTU
M. cardinalis 3	Ross 6675 UC	CA	CA 03	*M. cardinalis*	PB 99-048 WTU
M. cardinalis 4	Ahart 6581 UC	CA	CA 04	*M. cardinalis*	PB 99-044 WTU
M. cardinalis 5	Schemske, personal collection	CA	CA 05	*M. cardinalis*	PB 99-028 WTU
M. eastwoodiae	Welsh and Neese 22585 UTC	UT	CA 06	*M. cardinalis*	PB 99-026 WTU
M. lewisii Northern 1	Baird 3397 WTU	UT	CA 07	*M. cardinalis*	PB 99-023 WTU
M. lewisii Northern 2	Smith 2692 WTU	ID	CA 08	*M. cardinalis*	PB 99-031 WTU
M. lewisii Northern 3	Christy 760 ASU	WY	CA 09	*M. cardinalis*	PB 99-060 WTU
M. lewisii Northern 4*	D. Schemske, personal collection	MT	CA 10	*M. cardinalis*	AA LT4X3-9 WTU
M. lewisii Sierran 1	Taylor and Swanson 4961 JEPS	CA	CA 11	*M. cardinalis*	M. Tulig 1005 personal collection
M. lewisii Sierran 2	Osward and Ahart 3945 UC	CA	Mexico 12	*M. cardinalis*	Vickery 11315 UT
M. lewisii Sierran 3	Bacigalupi 6634 JEPS	CA	CA 13	*M. cardinalis*	PB 99-030 WTU
M. lewisii Sierran 4	Myrick 125 JEPS	CA	CA 14	*M. cardinalis*	PB 99-047 WTU
*M. rupestris**	Vickery 9102 UT	Mexico	AZ 15	*M. cardinalis*	PB 2001-01 WTU
M. verbenaceus	Van Devender 96-201 ARIZ	Mexico	AZ 16	*M. cardinalis*	PB 2001-02 WTU
M. verbenaceus	Windham 92720 COLO	UT	CO 01	*M. eastwoodiae*	PB 2000-46 WTU
M. nelsonii	Bell 17703 ASU	Mexico	UT 02	*M. eastwoodiae*	PB 2000-51 WTU
M. parishii	SES 98-61 CDA	CA	UT 03	*M. eastwoodiae*	PB 2000-55 WTU
M. bicolor	PB 98-068 WTU	CA	AZ 01	*M. verbenaceus*	PB 2000-60 WTU
M. filicaulis	PB 98-095 WTU	CA	AZ 02	*M. verbenaceus*	PB 2001-02 WTU
M. rubellus	PB 98-083 WTU	CA	AZ 03	*M. verbenaceus*	PB 2001-02 WTU
M. primuloides	PB 98-104 WTU	CA	UT 04	*M. verbenaceus*	PB 00-62 WTU
M. androsaceus	PB 98-023 WTU	CA	UT 05	*M. verbenaceus*	PB 2000-64 WTU
M. palmeri	PB 98-008 WTU	CA	Mexico 01	*M. nelsonii*	Vickery 6271 WTU
M. montiodes	SES 98-61 CDA	CA	Mexico 01*	*M. rupestris*	Vickery 9102 WTU
M. norrisii	PB 98-013 WTU	CA	WA 01	*M. lewisii* northern race	PB 98-129 WTU
M. floribundus	PB 98-125 WTU		WA 02	*M. lewisii* northern race	PB seed collection WTU
M. moschatus	Whittal 46 OSC		WA 03	*M. lewisii* northern race	PB seed collection WTU
M. guttatus	PB 98-121 WTU	CO	OR 04.1	*M. lewisii* northern race	PB 90-068 (1) WTU
M. wiensii	6273-4 Vickery WTU	Mexico	OR 04.2	*M. lewisii* northern race	PB 99-068 (2) WTU
M. alsinoides	Whittall 40 OSC		OR 05	*M. lewisii* northern race	PB 99-069 WTU
			MT 06*	*M. lewisii* northern race	D. Schemske, personal collection
			NCA 07.1	*M. lewisii* northern race	PB 2000-69 (1) WTU
			NCA 07.2	*M. lewisii* northern race	PB 2000-69 (3) WTU
			NCA 08	*M. lewisii* northern race	PB 2000-70 WTU
			CA 01.1	*M. lewisii* Sierran race	PB 99-050 (1) WTU
			CA 01.2	*M. lewisii* Sierran race	PB 99-050 (2) WTU
			CA 02	*M. lewisii* Sierran race	PB 99-052 WTU
			CA 03	*M. lewisii* Sierran race	PB 99-057 WTU
			CA 04	*M. lewisii* Sierran race	PB 99-059 WTU
			CA 05.1	*M. lewisii* Sierran race	PB 99-056 (1) WTU
			CA 05.2	*M. lewisii* Sierran race	PB 99-056 (2) WTU

TABLE 1. Continued.

ITS/ETS study			AFLP study		
Species/sample	Voucher/herbarium	Location	State/sample	Species	Voucher
			CA 05.3	*M. lewisii* Sierran race	PB 99-056 (3) WTU
			CA 06	*M. lewisii* Sierran race	PB 99-067 WTU
			CA 07	*M. lewisii* Sierran race	D. Schemske, personal collection
			CA 01.1	*M. parishii*	PB 99-043 (1) WTU
			CA 01.2	*M. parishii*	PB 99-043 (2) WTU
			CA 02	*M. parishii*	PB 99-029-1 WTU
			CA 03	*M. parishii*	SES 98-61 WTU
			CA 01	*M. bicolor*	PB 2000-15 WTU
			CA 01	*M. filicaulis*	PB 2000-02 WTU
			CA 01	*M. rubellus*	PB 98-083 WTU

tana State University, 1998; http://hordeum.oscs.montana.edu/genographer/) for visualization and scoring. AFLP loci were analyzed using the ''thumbnail'' option of Genographer, which allows comparison of signal strength at each locus for all samples. Band presence or absence was scored for each sample and recorded as a binary character. For the bulked DNA analysis, polymorphisms within species were not detectable because the samples were combined. Data matrices were constructed and analyzed using PAUP* 4.0b3a (Swofford 1998).

Relationships within *Erythranthe* were explored using parsimony and neighbor-joining (NJ) analyses. For analysis of the dataset with all individuals represented, parsimony and bootstrap searches were conducted as in the ITS/ETS analysis. For analysis of the bulked dataset, parsimony searches were conducted using a branch and bound search. Bootstrap values were calculated using 1000 replicate full branch and bound parsimony searches with MULTREES and TBR branch swapping. Distance estimates for the NJ analysis were calculated using the index of Nei and Li (1979).

Exploration of Character Changes and Ancestral State Reconstruction

Character data for extant taxa were obtained both from the literature (Hiesey et al. 1971; Vickery and Wullstein 1987; Sutherland and Vickery 1993; Thompson 1993; Vickery and Sutherland 1994; Bradshaw et al. 1998) and from measurements on greenhouse-grown populations of *M. parishii*. The following characters were studied: pollination syndrome (scored as a single character for heuristic purposes), nectar production, stamen length, pistil length, degree of reflex of upper and lower petals, and three petal color characters. The three petal color characters are (1) presence of carotenoids (yellow in color) in the upper petal, which is controlled by one gene, the yellow upper petal (YUP) locus (Hiesey at al. 1971, Bradshaw et al. 1998) in *M. cardinalis* and *M. lewisii*; (2) presence of carotenoids in the lower petal, a trait controlled by more than one locus in these two taxa (Hiesey et al. 1971); and (3) a simple description of corolla color. Corolla color has been shown by previous researchers to play a role in pollinator visitation (Vickery 1995). Continuous characters were converted to discrete, binary characters by constructing two bins for each character (Table 2) for further analyses. Models of character change were inferred on the optimal tree from the bulked DNA AFLP study in which each species was represented by one terminal taxon with the exception that *M. lewisii* was represented by both races. Outgroup relationships were determined in the ITS/ETS study. This tree, with added outgroups, is referred to as the input tree. Ancestral state reconstructions optimized using maximum parsimony were calculated using MacClade version 3.0 (Maddison and Maddison 1992). Ancestral pollination syndrome reconstructions, optimized using maximum likelihood, were performed using Discrete version 4.0 (Pagel 1994; http://www.ams.reading.ac.uk/zoology/pagel/), which employs a Markov transition model of evolutionary change. Branch lengths for the input tree were computed using parsimony branch lengths from the ITS/ETS study and also from the

TABLE 2. Character data used to reconstruct ancestral states. Each character was converted to a binary character using the following bins: Nectar production 0–1.1 μl, low (0); 7–17 μl, high (1). Stamen length 0–21 mm, short (0); 32–50 mm, long (1). Style length 0–24 mm, short (0); 32–47 mm, long (1). Petal reflection: 1, reflexed forward; 2, no reflex; 3, reflexed backwards. Carotenoids absent, 0; present, 1.

Species	Pollination syndrome	Nectar production in nature (μl)	Stamen length (mm)	Style length (mm)	Upper petal reflex (1–3)	Lower petal reflex (1–3)	Carotenoids in upper petal	Carotenoids in lower petal
M. lewisii (Sierras)	bee (0)	0.6 (0)	18.9 (0)	16.0 (0)	2	1.5	0	0
M. lewisii (Rockies)	bee (0)	0.97 (0)	20.2 (0)	23.3 (0)	2	2	0	0
M. cardinalis	hummingbird (1)	12.08 (1)	34 (1)	32.7 (1)	3	3	1	1
M. parishii	insect/self (0)	0 (0)	9 (0)	9.2 (0)	2	2	0	0
M. eastwoodiae	hummingbird (1)	1.1 (0)	32.8 (1)	33.6 (1)	2.8	2	1	0
M. verbenaceus	hummingbird (1)	7.27 (1)	37.4 (1)	38.3 (1)	3	2	1	1
M. nelsonii	hummingbird (1)	16.1 (1)	49.5 (1)	46.8 (1)	3	2	1	1
M. rupestris	hummingbird (1)	0.99 (0)	38.4 (1)	36.6 (1)	1	1	1	0
M. filicaulis	insect (0)	inferred (0)	inferred (0)	inferred (0)	2	2	1	1
M. rubellus	insect/self (0)	inferred (0)	inferred (0)	inferred (0)	2	2	0/1	0/1
M. bicolor	insect (0)	inferred (0)	inferred (0)	inferred (0)	2	2	0/1	0/1
M. primuloides	insect (0)	inferred (0)	inferred (0)	inferred (0)	2	2	1	1

Nei-Li distances on the neighbor-joining tree constructed using the bulked AFLP data (many outgroups were excluded on this tree because they were not sampled for AFLP data). Branches of zero length on the ITS/ETS tree were assigned the arbitrary length of 0.1 because Discrete will not accept zero branch lengths. Because more species were sampled, the ITS/ETS tree contains more outgroups than the AFLP tree. Outgroups included in the ITS/ETS tree were *M. primuloides*, *M. rubellus*, and *M. filicaulis*, whereas the AFLP tree included only *M. bicolor*. The effect of adding or subtracting outgroup species on ancestral state reconstruction was investigated.

To test hypotheses of whether switches in pollinators are consistent with a gradual or punctuational mode of change, we explored using different values for a branch-length scaling parameter (κ; Pagel 1994). A likelihood-ratio test was performed with $\kappa = 0$ (all the branch lengths are assumed to be unit length) and κ = the maximum-likelihood (ML) value. Trait evolution is consistent with a punctuational mode of change if κ (ML) is not significantly different from zero (Pagel 1994).

RESULTS

ITS/ETS Analysis

The total aligned length of the ITS region was 624 base pairs (bp) and the aligned ETS sequences were 380 bp in length. A limited number of short gaps (1–5 bp) were introduced to properly align the sequences. The ETS and ITS region are closely linked in the rDNA and results of the partition homogeneity test for ITS versus ETS showed that the datasets were not significantly different from random pairwise partitions of the data ($P = 0.62$). Trees were constructed for each DNA region separately and examined for hard topological incongruencies, and no well-supported incongruencies were found. Therefore, we combined the datasets in all analyses. The combined nrDNA dataset was 1004 bases in aligned length. Including all of the outgroups, 279 sites were variable and 180 were parsimony-informative. However, for the eight taxa within *Erythranthe*, only 15 parsimony-informative sites exist. Parsimony analyses for all taxa resulted in 85 most-parsimonious trees of length 432 (consistency index [CI] = 0.792, retention index [RI] = 0.873, rescaled consistency index [RC] = 0.691; Fig. 2).

Results from the analyses of the ITS and ETS results indicate the following: (1) If the desert species *M. parishii* is included, section *Erythranthe* forms a monophyletic group with a relatively high bootstrap (bs) value (87%). (2) A well-supported clade (100%) containing *M. bicolor*, *M. filicaulis*, and *M. rubellus* is sister to *Erythranthe*. The monophyly of the *Erythranthe* clade and the *M. bicolor*, *M. filicaulis*, and *M. rubellus* clade is also highly supported (99%). (3) *Mimulus primuloides* is strongly supported (99%) as sister to the clade containing *Erythranthe* and *M. bicolor*, *M. filicaulis*, and *M. rubellus*. (4) The number of inferred substitutions in ITS and ETS among species within *Erythranthe* is small, and branch lengths on the most-parsimonious trees are very short (0–13 steps). (5) Relationships within *Erythranthe* are generally unresolved. A poorly supported clade (54%) contains *M. rupestris*, *M. nelsonii*, *M. verbenaceus*, and *M. eastwoodiae*. Weak support exists (64%) for the sister relationship between *M. verbenaceus* and *M. eastwoodiae*. The relationships among *M. lewisii*, *M. cardinalis*, and *M. parishii* are unresolved. (6) At the population level, *M. lewisii* populations in the Rocky Mountains seem to be differentiated from those in the Sierra Nevada. The sequences for the *M. cardinalis* samples from Arizona are identical to the *M. cardinalis* from the Sierra Nevada and do not group within the *M. verbenaceus* samples from Arizona.

AFLP Analysis

A total of 474 AFLP fragments was scored for 53 accessions in the portion of the study in which data were gathered independently for each individual. The range of fragments collected per accession was 108–194 with a mean of 151.8 fragments. Neighbor-joining analyses of these data are presented in Figure 3 and led to the following results: (1) All

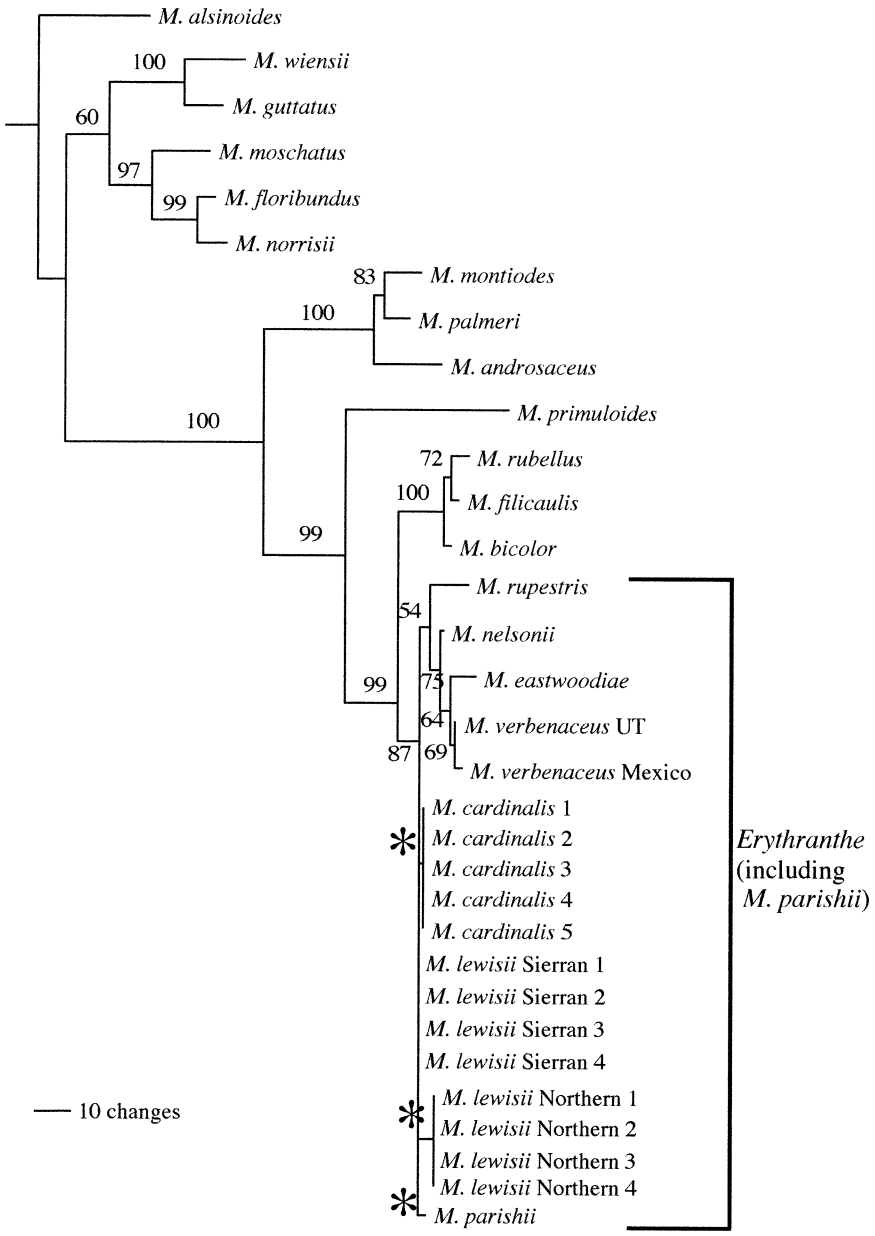

FIG. 2. One of the 85 most-parsimonious trees inferred using combined ITS and ETS data. Numbers subtending particular nodes indicate bootstrap support. Stars indicate nodes that collapse in the strict consensus tree.

of the accessions of each species, for which more than one individual was sampled, clustered together. Three have high levels of support (*M. cardinalis* 100%, *M. parishii* 100%, and *M. eastwoodiae* 96%). *Mimulus verbenaceus* was moderately supported as monophyletic (71%). (2) *Mimulus lewisii* was moderately supported as monophyletic (70%), but within *M. lewisii* are two well-supported clades (100% for both) corresponding to the two races recognized by Hiesey et al. (1971), which differ geographically and morphologically. Both of these clades are supported by several diagnostic AFLPs that are unique to each group. However, two *M. lewisii* individuals were not resolved to either of these two clusters (*M. lewisii* Northern race N CA 08 from the Siskiyou Mountains and *M. lewisii* Northern race WA 02 from the Cascades). The molecular data suggest that these individuals are intermediates between the two races, containing different combinations of the race-specific fragments. (3) *Erythranthe* (plus *M. parishii*) is strongly supported as monophyletic (98%). (4) Relationships among species in *Erythranthe* are moderately to well-supported, with bootstrap values ranging from 47% (*M. parishii*, *M. lewisii*, and *M. cardinalis*) to 98% (*M. eastwoodiae* and *M. verbenaceus*). A clade containing *M. rupestris*, *M. nelsonii*, *M. verbenaceus*, and *M. eastwoodiae* was recovered, with moderate support (65%). *Mimulus rupestris* and *M. nelsonii* are resolved as sister species (78%), and *M. cardinalis* and *M. lewisii* are together monophyletic (69%).

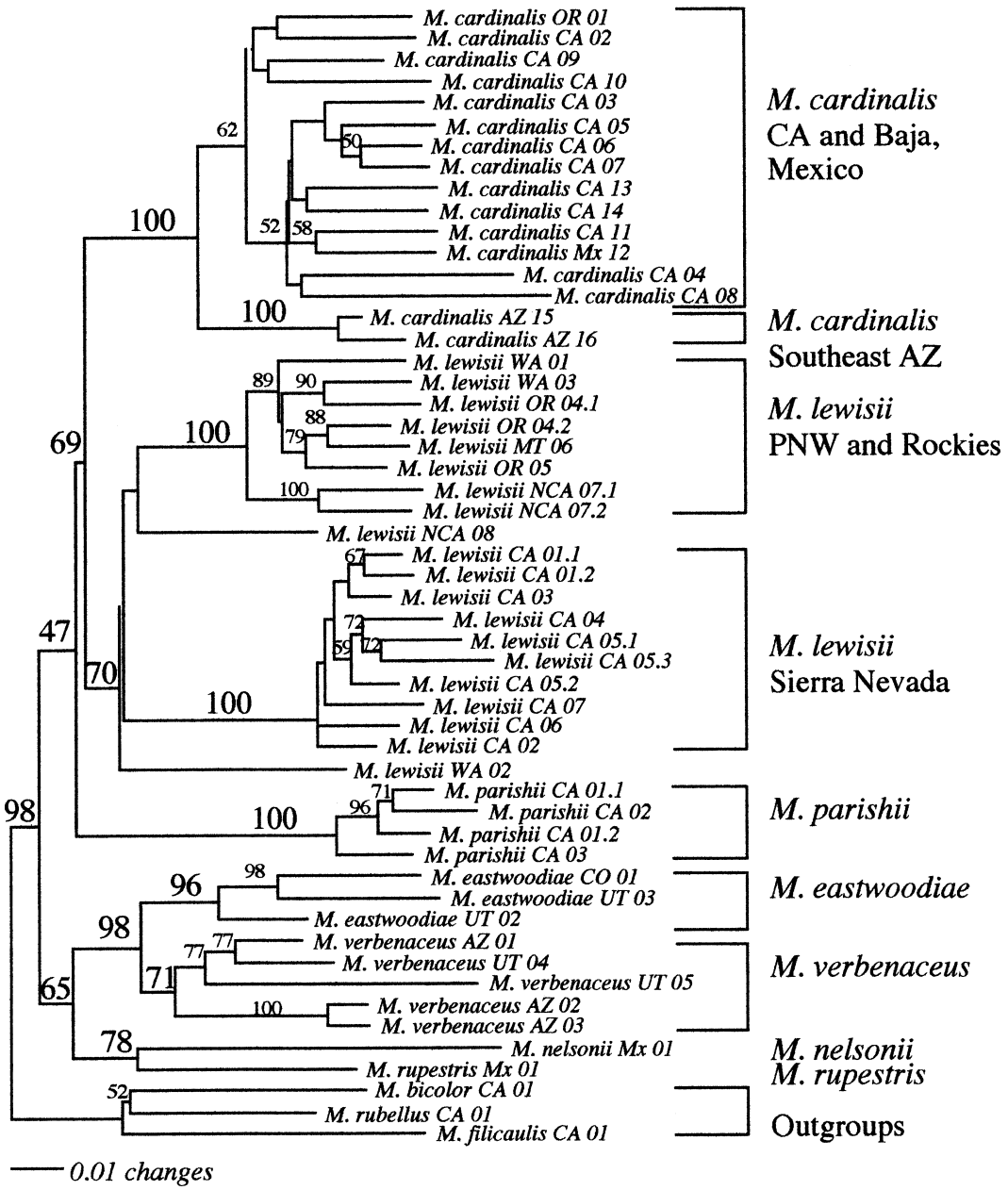

FIG. 3. Inferred neighbor-joining (NJ) tree for all 53 accessions using 474 AFLP fragments scored as present or absent. Numbers above the branches indicate NJ bootstrap values greater than 50.

The position of *M. parishii*, *M. rupestris*, and *M. verbenaceus* are not clearly resolved. Parsimony analyses of these same data recovered the same well-supported clades as the NJ analyses, but placed *M. parishii* as sister to the rest of the species in *Erythranthe*. This relationship, however, was poorly supported (bs $< 20\%$).

Because we had evidence for the monophyly of the species in *Erythranthe* and wished to obtain more data specifically on relationships among species, additional data were collected in which DNA from three individuals of each species (except for the uncommon *M. nelsonii* and *M. rupestris*, for which only one individual was available) was combined and patterns of AFLP fragments were compared between species. The two races of *M. lewisii* were each represented by three individuals. In this study, 452 fragments were scored for eight *Erythranthe* taxa and one outgroup. Neighbor-joining and parsimony analyses both recovered the same topology, with high levels of support for each node (Fig. 4). These analyses suggest the following relationships: (1) Species in *Erythranthe* are divided into two major clades, one containing species in the Sierras, the Pacific Northwest, and the Rocky Mountains (*M. parishii*, both subspecies of *M. lewisii*, and *M. cardinalis*), and one containing species in the arid Southwest and Mexico (*M. verbenaceus*, *M. eastwoodiae*, *M. nelsonii*, and *M. rupestris*). (2) The two races of *M. lewisii* are sister to each other, forming a monophyletic *M. lewisii*, which is

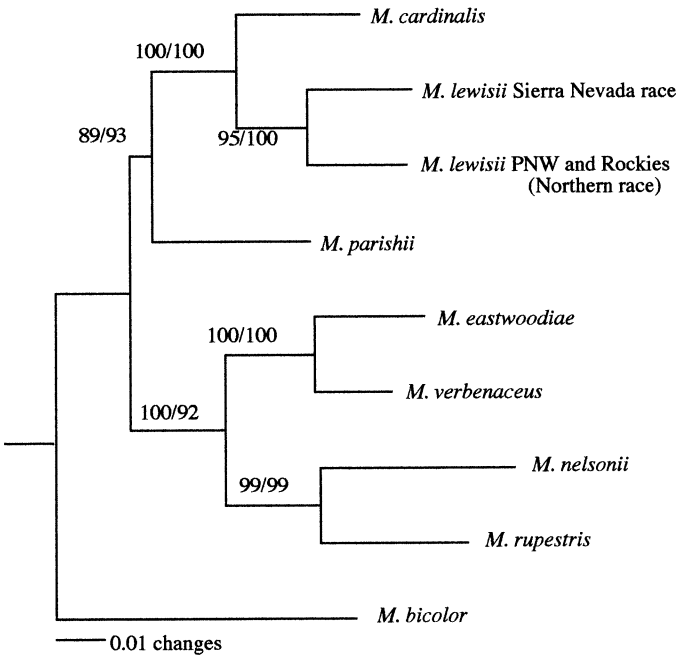

FIG. 4. Neighbor-joining tree estimated using 452 AFLP fragments obtained using bulked DNA from each taxon. The numbers subtending the nodes indicate bootstrap support estimates. The first number used neighbor-joining and the second number used parsimony.

sister to *M. cardinalis*. The *M. lewisii*/*M. cardinalis* clade is sister to *M. parishii*. (3) *Mimulus eastwoodiae* is sister to *M. verbenaceus*, and *M. rupestris* is sister to *M. nelsonii*. These two clades are sister to each other.

Character Analyses

Character states obtained from the literature and from our own measurements are presented in Table 2. A summary of ancestral state reconstructions optimized using maximum parsimony is shown in Figure 5. For pollination syndrome, the ancestor of *M. lewisii* and *M. cardinalis* is unambiguously reconstructed as being insect-pollinated, as is the ancestor of the *Erythranthe* clade (results not shown).

The following characters were found to be invariable among the hummingbird-pollinated taxa: stamen and pistil length (long), flower color (red), carotenoids in the upper petal (petal), and orientation of the upper petals (reflexed). Characters that varied among the hummingbird-pollinated taxa were nectar production, carotenoids in the lower petal, and orientation of lower petals.

Ancestral state reconstructions for pollination syndrome using maximum likelihood and ITS/ETS branch lengths are shown in Figure 6. Differences in likelihood of the two possible ancestral states were considered significant when they exceeded the conventional cutoff point of two log units (Edwards 1972; Pagel 1999). For these analyses, we assumed that the rate of transformation from hummingbird pollination to insect pollination was the same as the rate of transformation from insect pollination to hummingbird pollination ($\alpha = \beta$, see below). The maximum likelihood occurred with the branch-length scaling parameter $\kappa = 0.001$.

To test the hypothesis of whether switches in pollinators are consistent with a gradual or punctuational mode of change, a likelihood-ratio test was performed of $\kappa = 0$ and $\kappa =$ the maximum-likelihood (ML) value of 0.001 (likelihood values calculated assuming $\alpha = \beta$). The two likelihoods ($L = -12.32259$ and $L = -12.32261$ were not significantly different. For comparison, when $\kappa = 1$, $L = -15.082$.

The influence of outgroups was assessed by removing all but one of them, and recalculating ML values. The most likely ancestral states were identical to those calculated using four outgroups, although the probability of each reconstruction differed. The probability at six of the eight nodes decreased (a maximum of 14.6% decrease), whereas two nodes increased. The ML assignments of rates was $\alpha = 0.23$, $\beta = 0.00052$, similar to the results using four outgroups. Assuming that $\alpha = \beta$, the parameter κ was again estimated to be very low and not significantly different from zero. Ancestral pollination states, transformation rates, and the parameter κ were also estimated using the branch lengths derived from the NJ analysis of the bulked AFLP data but were nearly identical to the results using ITS/ETS branch lengths.

DISCUSSION

Usefulness of AFLP Data

Plant systematists possess few tools for effectively resolving species-level phylogenies in clades that have radiated relatively recently. Results from DNA sequence and AFLP analyses in *Mimulus* sect. *Erythranthe* indicate that AFLPs are useful for resolving phylogenies that are not well-resolved using DNA sequencing when sequence variation is limiting. Vickery and Wullstein (1987) used six different approaches to assess the relationships among the six traditional species in *Erythranthe*, including alpha taxonomy, numerical taxonomy, experimental hybridization, chemotaxonomy, allozyme comparisons, and DNA/DNA hybridizations. None of these approaches yielded a well-resolved estimate of relationships, and most emphasized the many similarities within *Erythranthe*. We analyzed relationships within *Erythranthe* by collecting data on two DNA regions commonly used in species-level systematics, the nrDNA ITS and ETS. Analyses of these data provided some insight into relationships and confirmed that this group of species has radiated relatively recently, but overall demonstrated little resolution. The rigorous application of AFLP data, however, provided a strongly-supported and fully-resolved phylogenetic hypothesis at the species level and added additional insights at the population level. The pattern of relationships suggested by sequence data is consistent with those inferred from AFLP data.

The use of AFLPs holds many advantages over other markers (e.g., RAPDs, microsatellites). They require no previous sequence knowledge, show very high levels of repeatability (Vos et al. 1995), and many loci can be assayed in a relatively short time due to the large number of fragments scored per gel (Hill et al. 1996; Mueller and Wolfenbarger 1999). AFLPs were also shown to generate tenfold more informative bands per primer in comparison to RAPDs (Sharma et al. 1996). The method of data collection we used includes an internal

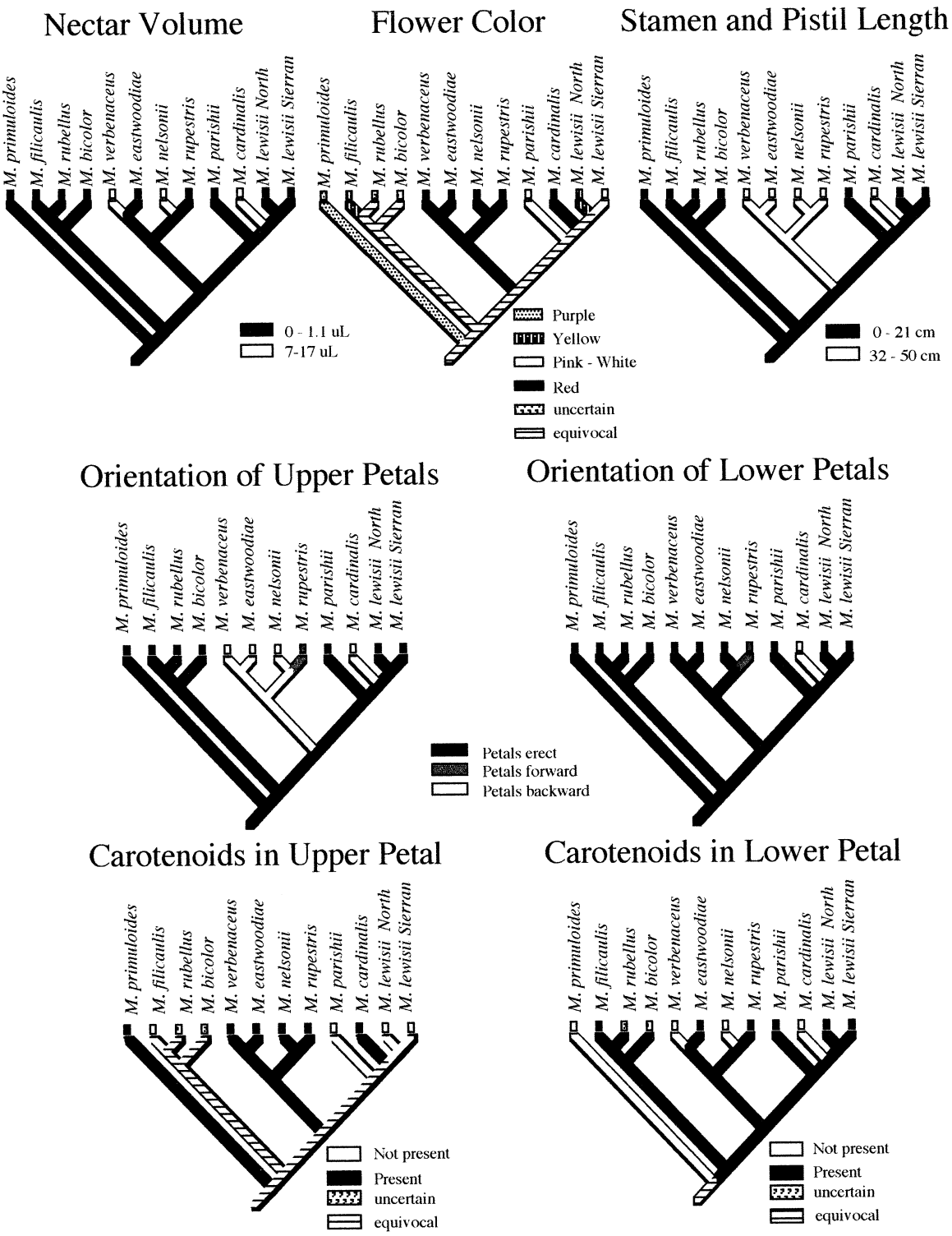

FIG. 5. Ancestral state of hummingbird pollination and individual characters associated with hummingbird pollination estimated using maximum parsimony. Tree used for ancestral state estimation is that inferred from AFLP data for the bulked DNA.

size standard in each lane, making band size calling much easier. Each lane is stored as an individual data file, which facilitates comparisons across different gels. The method is highly repeatable, relatively inexpensive, and three different primer combinations can be scored using one gel.

Analysis of AFLP data for phylogenetic reconstruction deserves more attention. Although further model development is encouraged, our own analyses suggest that with increasing amounts of AFLP data, all commonly used methods of reconstructing relationships with fragment data converge on

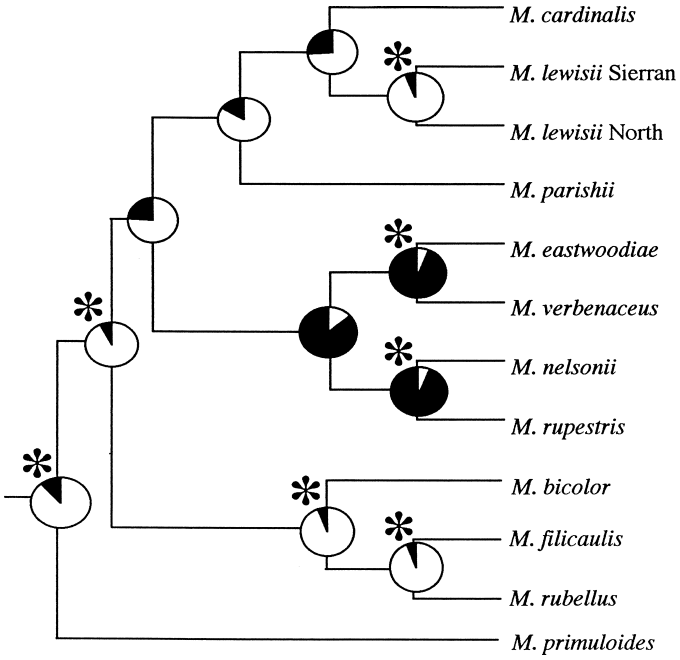

FIG. 6. Estimates of ancestral pollination states using maximum likelihood. Dark shading indicates hummingbird pollination and white indicates insect pollination. The shaded portion in each pie diagram corresponds to the calculated probability of that reconstruction. Stars indicate nodes in which one mode of pollination was significantly preferred. Branch lengths are not scaled in this diagram, because outgroups were not all sampled as part of the AFLP analysis.

the same tree and these estimates are robust. For example, estimates of relationships using maximum parsimony in which fragment gain and loss are weighted equally, and using Dollo parsimony, which makes the assumption that a character can be lost many times but gained only once, differ in the resolution of only one node (results not shown).

Relationships within Erythranthe

Species in *Erythranthe* have been a part of landmark studies on plant adaptation (Hiesey et al. 1971; Vickery 1978) and the subject of many biosystematic studies (Grant 1924; Hiesey et al. 1971; Vickery and Wullstein 1987). However, considerable confusion exists as to which species belong within *Erythranthe*. Both Grant (1924) and Greene (1885) suggested that *M. parishii* was closely related to *M. lewisii*, yet the absence of rigorous phylogenetic inference methods led subsequent workers to disregard their insight. Our results indicate that *Erythranthe* includes the following taxa: *M. cardinalis*, *M. lewisii*, *M. parishii*, *M. verbenaceus*, *M. eastwoodiae*, *M. nelsonii*, and *M. rupestris*.

The AFLP data resolve two major groups of species in *Erythranthe*; one contains *M. cardinalis*, *M. lewisii*, and *M. parishii*, and the other contains *M. verbenaceus*, *M. eastwoodiae*, *M. rupestris*, and *M. nelsonii*. The fact that *M. cardinalis* is more closely related to *M. lewisii* than it is to the other red-flowered, hummingbird-pollinated taxa has support from studies of crossing relationships (Vickery and Anderson 1967; Vickery 1969; Hiesey et al. 1971). The monophyly of the *M. verbenaceus*, *M. eastwoodiae*, *M. rupestris*, and *M. nelsonii* group is supported by the presence of anther lobes that are reflexed and horseshoe-shaped at the tip of the filament. This character is particularly useful to distinguish *M. cardinalis* from *M. verbenaceus* in Arizona, where there has been confusion in identifying these two taxa (Kearney and Peebles 1960). Support for this group can also be found in analyses of pollen morphology (Argue 1980).

The results of this study also shed light on the two races of *M. lewisii*. *Mimulus lewisii* is found from the Kings Canyon region in the southern Sierra Nevada mountains of California, north through the Cascade Ranges, to southern coastal Alaska, and east to Alberta, Idaho, Montana, Wyoming, Utah, and Colorado (Hiesey et al. 1971). Differences exist in corolla color, size, shape of leaves, and in stem and branching characteristics between *M. lewisii* populations in the Sierra Nevada (Sierra Nevada race) and those in the Cascade and Rocky Mountains (Northern race). Two studies have indicated that partial postzygotic barriers exist between these two systems of populations, putatively due to two pairs of reciprocal translocations. (Hiesey et al. 1971; Vickery and Wullstein 1987). The results of our analysis of 474 AFLP fragments indicate two very well-supported clusters (100%) that correspond to the two races. All of the *M. lewisii* from the Sierra Nevada fall into one cluster and all the *M. lewisii* from the Cascades and the Rockies fall into another, with two exceptions. *Mimulus lewisii* Northern race NCA 08 from the Siskiyou Mountains in northern California and *M. lewisii* Northern race WA 02 from Poe Mountain in the Cascades in Washington appear to be intermediate between the two races. The presence of intermediates between the races and the lack of substantive evidence for reproductive isolation in nature lead us to retain *M. lewisii* as one species at the present time.

Biogeography

Major biogeographic patterns within *Erythranthe* can be interpreted in light of the inferred phylogeny. All of the most closely related outgroups to *Erythranthe* are found in the Sierra Nevada of California. Assuming that present distributions reflect ancestral distributions, the most parsimonious geographic location for the common ancestor of the *Erythranthe* clade is in the Sierra Nevada. Early in the diversification of *Erythranthe*, a migration of the common ancestor of *M. verbenaceus*, *M. eastwoodiae*, *M. rupestris*, and *M. nelsonii* to deserts of the American Southwest and Mexico is inferred to have taken place. The distinct geographic ranges of these four species imply that allopatric speciation was the mechanism for further diversification in this region. The common ancestor of *M. lewisii*, *M. cardinalis*, and *M. parishii* is inferred to have existed in the Sierra Nevada where it subsequently diversified both morphologically and ecologically. The three extant species differ in habitat preferences, elevation, and pollinators (Hiesey et al. 1971; Thompson 1993; Bradshaw et al. 1995). *Mimulus lewisii* is inferred to have expanded its range from the Sierra Nevada to the north and east into the Cascade and Rocky Mountains. *Mimulus cardinalis* expanded its range east and south of the Sierra Nevada to include the coastal ranges, the transverse ranges, and the Baja peninsula. Populations of *M. cardinalis* have also es-

tablished in high elevations in southeastern Arizona, marking a second establishment of *Erythranthe* taxa in the southwestern deserts. The close relationship of the Arizona populations of *M. cardinalis* to those in the Sierra Nevada is confirmed in the ITS/ETS and AFLP studies. *Mimulus parishii* is distributed along streams in the desert mountain regions of southern California.

Reconstructing Ancestral Pollination Syndromes

Reconstructions of ancestral pollination syndromes using both parsimony and maximum likelihood suggest that the ancestor of the *Erythranthe* clade and the ancestor of *M. lewisii* and *M. cardinalis* were insect-pollinated. Given our estimates of ancestral states, hummingbird pollination evolved independently twice from insect-pollinated ancestors in *Erythranthe*. Statistical tests using maximum-likelihood models also indicate that pollinator transformations in *Erythranthe* more closely fit a model of punctuational evolution than a model of gradual evolution. This result is consistent with findings that QTLs of large effect are responsible for floral differences between *M. lewisii* and *M. cardinalis* (Bradshaw et al. 1995, 1998; Schemske and Bradshaw 1999).

Studies by Bradshaw and Schemske (Bradshaw et al. 1995, 1998; Schemske and Bradshaw 1999) have demonstrated that changes in floral morphology that affect pollinator visitation have a dramatic effect on reproductive isolation. Assuming that the ancestral state was insect pollination, both transformations to hummingbird pollination in this clade could be interpreted as adaptations to increase reproductive isolation in the diverging ancestral populations. However, selection for reproductive isolation can only occur when populations are sympatric. The common ancestor of *M. verbenaceus*, *M. eastwoodiae*, *M. rupestris*, and *M. nelsonii* is inferred to have existed in the deserts of the American Southwest and Mexico, whereas the ancestor of *M. lewisii*, *M. parishii*, and *M. cardinalis* clade is inferred to have existed in the Sierra Nevada. Thus, the transformation to hummingbird pollination in the ancestor of the *M. verbenaceus*, *M. eastwoodiae*, *M. rupestris*, and *M. nelsonii* clade was more likely due to selection for the most effective pollinator (Stebbins 1970) than for reproductive isolation per se.

Characters Associated with Hummingbird Pollination in Erythranthe

In addition to estimating the ancestral pollination syndrome, we separately estimated the ancestral state of individual characters putatively associated with hummingbird pollination to gain insight into the coevolution of these traits. Overall, our results show that the characters that are consistently correlated with hummingbird pollination in *Erythranthe* are exerted styles and stamens, red corollas, and reflexed upper petals, whereas high nectar production and reflexed lower petals were not consistent. The fact that long stamens and pistils were consistently and exclusively found in all hummingbird-pollinated species was expected. Both of these morphological traits are important for the effective removal of pollen from the anther and the deposition of pollen on the stigma, and thus directly influence fitness. Flower color in *Erythranthe* and its sister clade varies considerably between species, yet red corollas always exist in the hummingbird-pollinated taxa, consistent with the results of Schemske and Bradshaw (1999), who found that flower color contributes to pollinator-mediated reproductive isolation in *M. lewisii* and *M. cardinalis*, and the hypotheses for the evolution of hummingbird pollination through Batesian mimicry (Bleiweiss 2001).

Schemske and Bradshaw (1999) found an allele that increases nectar production increases the rate of hummingbird pollination twofold when homozygous. However, nectar production, in both the greenhouse and in nature, varied in *Erythranthe* more than tenfold (Vickery and Sutherland 1994). Two of the lowest producers of nectar were hummingbird-pollinated *M. eastwoodiae* and *M. rupestris*, both of which had values similar to the bee-pollinated *M. lewisii*. The common ancestor of the *M. verbenaceus*, *M. eastwoodiae*, *M. rupestris*, and *M. nelsonii* clade is estimated using parsimony to have produced low amounts of nectar. One extra step is required if the high nectar production evolved in the ancestor of this clade and was subsequently lost twice.

One interpretation of the low nectar values in *M. eastwoodiae* and *M. rupestris* is that they are functioning as Batesian mimics; attracting hummingbirds with red, tubular corollas but offering no nectar reward. Relative to other hummingbird-pollinated plants, Batesian mimics are expected to be rare. Consistent with the mimicry hypothesis, *M. rupestris* is exceedingly rare, known from only one population. *Mimulus eastwoodiae* is relatively infrequent in hanging gardens throughout the canyon country of southeastern Utah, northeastern Arizona, and southwestern Colorado. Found in similar ecological conditions are the putatively hummingbird-pollinated *Castilleja chromosa*, *Ipomopsis aggregata*, *Penstemon barbatus*, and *P. eatonii*. The frequency of low nectar populations of *M. eastwoodiae* should be further investigated. A second interpretation is that *M. eastwoodiae* and/or *M. rupestris* maintain the plesiomorphic condition for nectar production.

The phylogenetic estimation of the transformation to reflexed upper petals is correlated with the switch in pollination syndromes, whereas reflexed lower petals are not so correlated. This result was somewhat surprising given that the lower petals are those that would be involved in forming a landing platform for bees. Flowers with a landing platform are reported to be preferred by bumblebees (Percival 1979). Sutherland and Vickery (1993) report that hummingbirds show a preference for reflexed petals.

In conclusion, the common ancestor of *M. cardinalis* and *M. lewisii* is estimated to have been insect-pollinated, with a relatively short style and stamen, no carotenoids in the lower petal, erect upper and lower petals, and relatively low nectar volumes. The reconstruction of flower color and carotenoid accumulation is ambiguous. In many ways, then, the common ancestor would be expected to look similar to extant *M. lewisii*. The common ancestor of the clade comprising *M. verbenaceus*, *M. eastwoodiae*, *M. rupestris*, and *M. nelsonii* is estimated to have been hummingbird-pollinated, possessing a relatively long style and stamen, a red corolla due to the presence of carotenoids in the upper petal (but lacking carotenoids in the lower petal), upper petals reflexed back-

wards, lower petals erect, and producing low amounts of nectar.

The origin of hummingbird pollination in the lineage leading to *M. cardinalis* is consistent with an origin via Mullerian mimicry. Hummingbird pollination in the *M. verbenaceus*, *M. eastwoodiae*, *M. rupestris*, and *M. nelsonii* clade is consistent with an origin via Batesian mimicry followed by the evolution of increased nectar production and, as population numbers grew, a switch to Mullerian mimicry. In addition to experimental manipulations, results from other phylogenetic studies with explicit character state reconstruction are necessary before general conclusions about the relative importance of individual characters within the suite of traits associated with hummingbird pollination can be made.

Acknowledgments

We thank A. Angert, A. Colwell, A. Denton, D. Schemske, S. Schoenig, M. Tulig, and R. Vickery, Jr. for plant material and H. D. Bradshaw, Jr., M. Pagel, P. A. Reeves and D. Schemske for helpful discussions. Funding was provided by grants to PMB from the American Society of Plant Taxonomists and National Science Foundation grant DEB 9727025 to RGO.

Literature Cited

Argue, C. L. 1980. Pollen morphology in the genus *Mimulus* (Scrophulariaceae) and its taxonomic significance. Am. J. Bot. 67: 68–87.

Avise, J. C. 1994. Molecular markers, natural history and evolution. Chapman and Hall, New York.

Baldwin, B. G. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. Mol. Phylogenet. Evol. 1:3–16.

Baldwin, B. G., and S. Markos. 1998. Phylogenetic utility of the external transcribed spacer (ETS) of 18S–26S rDNA: Congruence of ETS and ITS trees of *Calycadenia* (Compositae). Mol. Phylogenet. Evol. 10:449–463.

Baldwin, B. G., M. J. Sanderson, J. M. Porter, M. F. Wojciechowski, C. S. Campbell, and M. J. Donoghue. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. MO. Bot. Gard. 82:247–277.

Beardsley, P. M., and R. G. Olmstead. 2002. Redefining Phrymaceae: the placement of *Mimulus*, tribe Mimuleae, and *Phryma*. Am. J. Bot. 89:1093–1102.

Bleiweiss, R. 2001. Mimicry on the QT(L): genetics of speciation in *Mimulus*. Evolution 55:1706–1709.

Bradshaw, H. D., Jr., S. M. Wilbert, K. G. Otto, and D. W. Schemske. 1995. Genetic mapping of floral traits associated with reproductive isolation in monkeyflowers (*Mimulus*). Nature 376: 762–765.

Bradshaw, H. D., Jr., K. G. Otto, B. E. Frewen, J. K. McKay, and D. W. Schemske. 1998. Quantitative trait loci affecting differences in floral morphology between two plant species of monkeyflower (*Mimulus*). Genetics 149:367–382.

Brown, J. H., and A. Kodrick-Brown. 1979. Convergence, competition, and mimicry in a temperate community of hummingbird-pollinated flowers. Ecology 60:1022–1035.

Bruneau, A. 1997. Evolution and homology of bird pollination syndromes in *Erythrina* (Leguminosae). Am. J. Bot. 84:54–71.

Caicedo, A. L., O. Gaitan, M. C. Duque, O. Toro Chica, D. G. Debouck, and J. Thome. 1999. AFLP fingerprinting of *Phaseolus lunatus* L. and related wild species from South America. Crop Sci. 39:1497–1507.

Cunningham, C. W., K. E. Omland, and T. H. Oakley. 1998. Reconstructing ancestral character states: a critical reappraisal. Trends Ecol. Evol. 13:361–366.

Doyle, J. J., and J. L. Doyle. 1987. A rapid isolation procedure for small quantities of leaf tissue. Phytochem. Bull. 19:11–15.

Edwards, A. W. F. 1972. Likelihood. Cambridge Univ. Press, Cambridge, U.K.

Farris, J. S., M. Kallersjö, A. G. Kluge, and C. Bult. 1994. Testing significance of incongruence. Cladistics 10:315–319.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791.

Ganders, F. R., M. Berbee, and M. Pirseyedi. 2000. ITS base sequence phylogeny in *Bidens* (Asteraceae): evidence for the continental relatives of Hawaiian and Marquesan *Bidens*. Syst. Bot. 25:122–133.

Ghebrehiwet, M., B. Bremer, and M. Thulin. 2000. Phylogeny of tribe Antirrhineae (Scrophulariaceae) based on morphological data and *ndh*F sequence data. Plant Syst. Evol. 220:223–239.

Givnish, T. J., T. M. Evans, M. L. Zjhra, T. B. Patterson, P. E. Berry, and K. J. Sytsma. 2000. Molecular evolution, adaptive radiation, and geographic diversification in the amphiatlantic family Rapateaceae: Evidence from ndhF sequences and morphology. Evolution 54:1915–1937.

Grant, A. L. 1924. A monograph of the genus *Mimulus*. Ann. MO. Bot. Gard. 11:99–389.

Grant, K. A. 1966. A hypothesis concerning the prevalence of red coloration in California hummingbird flowers. Am. Nat. 100: 85–97.

Grant, K. A., and V. Grant. 1968. Hummingbirds and their flowers. Columbia Univ. Press, New York.

Greene, E. L. 1885. Studies in the botany of California and parts adjacent. I. Bull. Calif. Acad. Sci. 1:66–127.

Hiesey, W. M., M. A. Nobs, and O. Bjorkman. 1971. Carnegie Inst. Washington Publ. 628:1–213.

Hill, M., H. Witsenboer, M. Zabeau, P. Vos, R. Kesseli, and R. Michelmore. 1996. PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca* spp. Theor. Appl. Genet. 93:1202–1210.

Hodges, S. A. 1997. A rapid radiation via a key innovation in *Aquilegia*. Pp. 391–405 *in* T. Givnish and K. Sytsma, eds. Molecular evolution and adaptive radiations, Cambridge Press, Cambridge, U.K.

Kearney, T. H. and R. H. Peebles. 1960. Arizona flora. Univ. of California Press, Berkeley, CA.

Maddison, D. R. 1991. The discovery and importance of multiple islands of most-parsimonious trees. Syst. Zool. 40:315–328.

Maddison, W. P., and D. R. Maddison. 1992. MacClade: analysis of phylogeny and character evolution. Ver. 3.0. Sinauer, Sunderland, MA.

McDade, L. A. 1992. Pollinator relationships, biogeography, and phylogenetics. Bioscience 42:21–26.

Mueller, U. G., and L. L. Wolfenbarger. 1999. AFLP genotyping and fingerprinting. Trends Ecol. Evol. 14:389–394.

Nei, M., and W. H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA. 76:5269–5273.

O'Kane, S. L., Jr.,and B. A. Schaal. 1998. Phylogenetics of *Lopezia* (Onagraceae): Evidence from chloroplast DNA restriction sites. Syst. Bot. 23:5–20.

Olmstead, R. G., C. W. dePamphilis, A. D. Wolfe, N. D. Young, W. J. Elisens, and P. A. Reeves. 2001. Disintegration of the Scrophulariaceae. Am. J. Bot. 88:348–361.

Pagel, M. 1994. Detecting correlated evolution on phylogenies: a general method for the comparative analysis of discrete characters. Proc. R. Soc. Lond. B. Biol. Sci. 255:37–45.

———. 1999. The maximum likelihood approach to reconstructing ancestral character states of discrete characters on phylogenies. Syst. Biol. 48:612–622.

Percival, M. S. 1979. Floral biology. Pergamon, Oxford, U.K.

Schemske, D. W., and H. D. Bradshaw, Jr. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). Proc. Natl. Acad. Sci. USA. 96:11910–11915.

Sharma, S. K., M. R. Knox, T. H. N. Ellis. 1996. AFLP analysis of the diversity and phylogeny of *Lens* and its comparison with RAPD analysis. Theor. Appl. Genet. 93:751–758.

Stebbins, G. L. 1970. Adaptive radiation of reproductive charac-

teristics in angiosperms. I. Pollination mechanisms. Annu. Rev. Ecol. Syst. 1:307–326.
———. 1989. Adaptive shifts toward hummingbird pollination. Pp. 39–60 *in* J. H. Bock and Y. B. Linhart, eds. The evolutionary ecology of plants. Westview Press, Boulder, CO.
Sutherland, S. D., and R. K. Vickery, Jr. 1993. On the relative importance of floral color, shape, and nectar rewards in attracting pollinators to *Mimulus*. Great Basin Nat. 53:107–117.
Swofford, D. L. 1998. PAUP*: phylogenetic analysis using parsimony. * Sinauer, Sunderland, MA.
Thompson, D. M. 1993. *Mimulus*. Pp.1037–1051 *in* J. C. Hickman, ed. The Jepson manual. Univ. of California Press, Berkeley, CA.
Vickery, R. K. Jr. 1969. Crossing barriers in *Mimulus*. J. Jpn. Genet. 44(suppl 1):325–336.
———. 1978. Case studies in the evolution of species complexes in *Mimulus*. Evol. Biol. 11:404–506.
———. 1995. Speciation in *Mimulus*, or, can a simple flower color mutant lead to species divergence? Great Basin Nat. 55:177–180.
Vickery, R. K., Jr.,and D. G. Anderson. 1967. Experimental hybridizations in the genus *Mimulus* (Scrophulariaceae). VI. Section *Erythranthe*. Proc. Utah Acad. Sci. Arts Lett. 44:321–333.
Vickery, R. K., Jr.,and S. D. Sutherland. 1994. Variance and replenishment of nectar in wild and greenhouse populations of *Mimulus*. Great Basin Nat. 54:212–227.
Vickery, R. K., Jr.,and B. M. Wullstein. 1987. Comparison of six approaches to the classification of *Mimulus* sect. *Erythranthe* (Scropulariaceae). Syst. Bot. 12:339–364.
Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407–4414.
Weller, S. G., and A. K. Sakai. 1999. Using phylogenetic approaches for the analysis of plant breeding system evolution. Annu. Rev. Ecol. Syst. 30:167–199.
Wolfe, A. D., and A. Liston. 1998. Contributions of PCR-based methods to plant systematics and evolutionary biology. Pp. 43–86 *in* D. E. Soltis, P. S. Soltis, and J. J. Doyle, eds. Molecular systematics of plants II DNA sequencing. Kluwer, Boston, MA.
Xu, F., and M. Sun. 2001. Comparative analysis of phylogenetic relationships of grain amaranths and their wild relatives (*Amaranthus*; Amaranthaceae) using internal transcribed spacer, amplified fragment length polymorphism, and double-primer fluorescent intersimple sequence repeat markers. Mol. Phylogenet. Evol. 21:372–387.
Xu, R. Q., N. Tomooka, and D. A. Vaughan. 2000. AFLP markers for characterizing the Azuki bean complex. Crop. Sci. 40: 808–815.
Zhang, L-B., H. P. Comes, and J. W. Kadereit. 2001. Phylogeny and quaternary history of the European montane/alpine endemic Soldanella (Primulaceae) based on ITS and AFLP variation. Am. J. Bot. 88:2331–2345.

Corresponding Editor: J. Willis