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REDEFINING PHRYMACEAE: THE PLACEMENT OF *MIMULUS*, TRIBE MIMULEAE, AND *PHRYMA*[1]

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Chloroplast *trnL/F* and nuclear ribosomal ITS and ETS sequence data were used to analyze phylogenetic relationships among members of tribe Mimuleae (Scrophulariaceae) and other closely related families in Lamiales. The results of these analyses led to the following conclusions. (1) The Australian genera *Glossostigma* and *Peplidium* and the taxonomically isolated *Phryma* join four genera of tribe Mimuleae to form a well-supported clade that is distinct from other families in the Lamiales. We refer to that clade as the subfamily Phrymoideae. (2) The genera *Mazus* and *Lancea* (tribe Mimuleae) together form a well-supported clade that we recognize as the subfamily Mazoideae. Mazoideae is weakly supported as sister to Phrymoideae. We assign Mazoideae and Phrymoideae to a redefined family Phrymaceae. (3) *Mimulus* is not monophyletic, because members of at least six other genera have been derived from within it. In light of the molecular evidence, it is clear that species of Phrymaceae (about 190 species) have undergone two geographically distinct radiations; one in western North America (about 130 species) and another in Australia (about 30 species). Phylogenetic interpretations of morphological evolution and biogeographical patterns are discussed.

Key words: ETS; ITS; Mimuleae; *Mimulus*; *Phryma*; Phrymaceae; Scrophulariaceae; *trnL/F*.

Species in the genus *Mimulus* have become model systems for the study of evolutionary processes in nature. The most intensely studied species are part of the radiation of *Mimulus* in western North America. North American *Mimulus* was established for the study of evolution by the classic work of scientists at the Carnegie Institution. They investigated biosystematics, genetics, and physiological ecology in sect. *Erythranthe* (Hiesey, Nobs, and Bjorkman, 1971). Research on *Mimulus* has continued with studies of inbreeding depression (Carr and Dudash, 1996; Carr, Fenster, and Dudash, 1997; Karron et al., 1997; Dudash and Carr, 1998; Willis, 1999) the genetics of speciation (Bradshaw et al., 1995, 1998), mating system evolution (Leclerc-Potvin and Ritland, 1994), and the ecological effects of hybridization (Beeks, 1962; Waayers, 1996).

As currently described, *Mimulus* contains approximately 120 species, of which some 75% occur only in western North America. However, *Mimulus* is worldwide in distribution. Four species exist in Australia (Grant, 1924; Barker, 1982) (one of them also occurs in South Africa and India), 10 in Chile (von Bohlen, 1995a), approximately 19 in Mexico (Grant, 1924; Vickery, 1997), 4 in the Himalayas (Yamazaki, 1993), 1 in Madagascar, and 2 in eastern North America. The relationships among western North American *Mimulus*, *Mimulus* species distributed outside western North America, and several genera putatively closely related to *Mimulus* remain uncertain.

Dumortier (1829) first proposed tribe Mimuleae (Scrophulariaceae). Von Wettstein (1891) placed *Mimulus* with *Mazus*, *Dodartia*, *Monttea*, *Melosperma*, and *Lancea* in subtribe Mimulinae of tribe Gratioleae. *Leucocarpus*, *Hemichaena*, and *Berendtiella* were placed in tribe Scrophularieae. Pennell (1920) redefined tribe Mimuleae to contain *Leucocarpus* and *Mimulus*, though in subsequent works, Pennell placed Mimuleae in tribe Gratioleae (Pennell, 1935, 1947). Pennell (1935) also transferred *Hemichaena* and *Berendtiella* to tribe Gratioleae, noting a similarity in floral structure to *Mimulus*. Burtt (1965) reestablished Mimuleae as a tribe, diagnosed on the presence of two characters: (1) tubular, toothed calyces and (2) bilamellate stigmas that are receptive only on the inner surface and that close together with contact. Thieret (1954, 1967) suggested the removal of *Melosperma* and *Monttea* to Melospermeae. This left tribe Mimuleae sensu Argue (1984) composed of *Mimulus* (125 species), *Berendtiella* (5 species), *Hemichaena* (1 species), *Leucocarpus* (1 species), *Dodartia* (1 species), *Lancea* (2 species), and *Mazus* (25 species). The relationships among *Mimulus* and other members of Mimuleae are poorly understood. In his analysis of pollen, Argue (1980, 1984) found no character or combination of characters that separates *Dodartia*, *Lancea*, *Leucocarpus*, *Mazus*, or *Mimulus*.

Barker (1982) considered the Australian genera *Glossostigma*, *Peplidium*, and *Elacholoma* (subtribe Limoselleae of tribe Gratioleae, sensu Bentham [1876]) to be members of Mimuleae. Similar to the traditional members of Mimuleae, these Australian genera possess tubular, toothed calyces, though in *Glossostigma* the calyx has three or four lobes instead of the typical five lobes. The stigmas of these Australian genera are structurally different from those of species traditionally assigned to Mimuleae. In the monotypic *Elacholoma*, the stigma lobes are relatively long and are receptive over most of their length (Barker, 1982). The stigmas of *Glossostigma* and *Peplidium* contain one large and one vestigial stigma lobe. The large lobe covers the mouth of the corolla and is receptive only on the outer surface (Barker, 1982). Upon being touched, this large stigma lobe moves from covering the mouth to pressing against the upper corolla lip, exposing the anthers. In order to assess the homology of these stigmatic movements to the touch-sensitive stigmatic movements in traditional Mimuleae, a sound phylogenetic hypothesis is needed.

Though much has been learned about processes of evolution in *Mimulus*, the systematic placement of the genus and the relationships among species within it remain unresolved. Molecular studies have demonstrated that the traditionally rec-

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ognized Scrophulariaceae are not monophyletic (Olmstead and Reeves, 1995; Olmstead et al., 2001). In their analysis of Scrophulariaceae using three chloroplast genes, Olmstead et al. (2001) found that the single representative of *Mimulus* formed the sister group to Orobanchaceae and *Paulownia* in some trees and to Lamiaceae in others. Additional taxa were not sampled within *Mimulus* or tribe Mimuleae. Olmstead et al. (2001) dismembered the traditional Scrophulariaceae but did not reassign *Mimulus* to any clade of family rank.

An additional intriguing result of molecular analyses of Lamiales is the close relationship between *Mimulus* and the taxonomically isolated *Phryma* (Phrymaceae) (R. G. Olmstead, unpublished manuscript). *Phryma* is monotypic with a disjunct distribution in eastern Asia and eastern North America. The taxonomic position of *Phryma* has been unsettled for years: some authors place it in Verbenaceae (e.g., Cronquist, 1981) and others in its own family Phrymaceae (e.g., Schauer, 1847; Moldenke, 1971; Lu, 1990). *Phryma* has a distinctive pseudomonomerous gynoecium, which develops into a one-seeded fruit (Chadwell, Wagstaff, and Cantino, 1992). A close relationship between *Phryma* and *Mimulus* has never been suggested.

Relationships within *Mimulus* have also been a source of taxonomic controversy. Grant (1924) divided the approximately 120 species into two subgenera and ten sections. Subgenus *Mimulus* (*Synplacus* sensu Grant) is based on the character of the placentae being firmly united to form a central column or separating only at the apex. Taxa within subgenus *Schizoplacus* possess placentae that are divided to the base. The subgeneric assignments made by Grant have not been challenged, although differing views on relationships among and within sections have been proposed and some sections have been elevated to generic rank (McMinn, 1951; Pennell, 1951). Vickery (1969) recognized seven major sections in *Mimulus* (*Mimulus*, *Erythranthe*, *Simiolus*, *Paradanthus*, *Eunanus*, *Oenoe* and *Diplacus*) and five monotypic sections. Thompson's (1993) treatment of *Mimulus* in California follows Vickery, but reassigns *M. mohavensis* (monotypic sect. *Mimulastrum*) and *M. pictus* (monotypic sect. *Pseudoenoe*) to sect. *Mimulastrum* and places *M. pygmaeus* (monotypic sect. *Microphyton*) in sect. *Oenoe*. The monophyly of some of the sections is suspect, because other sections have been suggested to be derived from within them (Grant, 1924; D. Thompson, personal communication).

The goals of this study are to: (1) develop a rigorous phylogenetic hypothesis for the higher level relationships of *Mimulus* and other members of the tribe Mimuleae, (2) examine the relationship of *Mimulus* to putatively closely related genera, (3) test the hypothesis of monophyly of the genus *Mimulus*, (4) analyze subgeneric and sectional relationships within *Mimulus*, and (5) assess biogeographical relationships and morphological character changes in a phylogenetic context.

We report the results of analyses of DNA sequences from both the chloroplast and nuclear genome. Data from the chloroplast genome come from the leucine (*trnL*) intron and the intergenic spacer between *trnL* and *trnF* (*trnL/F*) (Gilley and Taberlet, 1994). This region has been shown in previous studies to provide good phylogenetic resolution within families and genera in the Lamiales (McDade and Moody, 1999). The nuclear genome is represented by sequences of both the internal transcribed spacer (ITS) and external transcribed spacer (ETS) regions (Baldwin et al., 1995; Baldwin and Markos, 1998) of nuclear rDNA. We collected nuclear genome data for two reasons: (1) to compare phylogenetic hypotheses from different genomes and (2) to help resolve more distal portions of the phylogeny. Our expectation in starting this project was that the substitution rate of *trnL/F*, ETS, and ITS would overlap, but overall the *trnL/F* region would have more slowly evolving sites. This information would be useful for resolving the deeper nodes in the phylogeny. The ETS and ITS would show higher overall substitution rates that would aid in resolving the distal portions of the phylogeny.

MATERIALS AND METHODS

Taxon sampling—All genera within the traditionally described tribe Mimuleae were sampled except *Dodartia*, a monotypic genus from Russia, and the monotypic Australian genus *Elacholoma* (Table 1). The Australian genera *Glossostigma* and *Peplidium* were sampled, as was *Phryma* from eastern North America and China. Within *Mimulus*, we included at least two representatives of each of the seven major sections. An attempt was also made to sample broadly across the geographical distribution of *Mimulus*. Species from western North America, eastern North America, Chile, Australia, and China were included. Molecular systematic analyses of nearly every species of *Mimulus* currently underway (P. Beardsley, unpublished data) indicate that our sample of species within *Mimulus* results in the same pattern of relationships as denser sampling. Also represented are members of the clades identified by Olmstead et al. (2001) as most closely related to *Mimulus*, including Paulowniaceae, Lamiaceae, Orobanchaceae, Pedaliaceae, and Acanthaceae. Some of the *trnL/F* and ITS sequences for the preceding families were obtained from GenBank. Species used in this study, voucher specimens, and GenBank accession numbers are listed on the Botanical Society of America website (http://ajbsupp.botany.org/v89/). A representative of Veronicaceae, *Mohavea breviflora*, was used as an outgroup in all analyses, a choice that is supported by previous molecular analyses of Lamiales (Olmstead et al., 2001).

Molecular methods—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, California, USA).

The *trnL/F* region was amplified using the *trn*-c and *trn*-f primers (Taberlet et al., 1991). We had difficulty amplifying both the intron and spacer as one fragment for three species (*Mimulus uvedaliae*, *Lancea tibetica*, and *Berendtiella rugosa*). For these taxa, the intron and spacer were amplified separately, using the *trn*-c and *trn*-d primers to amplify the *trnL/F* intron and *trn*-e and *trn*-f to amplify the spacer. The entire ITS region was amplified using the its4 and its5 primers (Baldwin, 1992). In *Hemichaena fruiticosa* and *Mimulus gracilis*, we had difficulty amplifying the entire fragment so the ITS1 and ITS2 were amplified separately, using the its5 and its2 primers for ITS1 and the its3 and its4 primers for ITS2. To amplify a portion of the 3′ end of the ETS, we used the 3′ 18S-IGS primer of Baldwin and Markos (1998). The 5′ primer, named ETS-B (5′-ATAGAGCGCGTGAGTGGTG-3′) (A. Yen, University of Washington, unpublished manuscript) was designed using *Mimulus* sequences as a reference. The polymerase chain reaction (PCR) conditions for all three DNA regions were as follows: 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The PCR products were purified using Qiagen Qiaquick spin-columns according to the manufacturer's protocol.

Sequences of both strands of the PCR product were generated on an ABI 377 (Applied Biosystems, Foster City, California, USA). To improve sequence quality for the *trnL/F* region of all sampled taxa, the internal primers of Taberlet et al. (1991), *trn*-d and *trn*-e, were used for sequencing as were two primers that we designed using *Mimulus* sequences as a reference, *trnL*-2C (5′-ATCGGTAGACGCTACGGACT-3′) and trnL-2F (5′-CGGGATAGCTCAGCTGGTAG-3′) that are just internal to *trn*-c and *trn*-f, respectively. The ITS was sequenced using the external PCR primers, its4 and its5, and the two internal primers, its2 and its3. The ETS was sequenced using the 18S-E primer of Baldwin and Markos (1998), which is slightly internal to 18S-IGS primer, and the *Mimulus* specific ETS-B primer. Electropherograms for each region were compiled and compared using the program Sequencher version 3.0

TABLE 1. Traditional classification of taxa assigned to Phrymaceae in this study. Classification is to tribe for all genera and to subgenus and section for species of *Mimulus*.

Taxon	Tribe	Subgenus	Section
Berendtiella rugosa	Mimuleae		
Glossostigma drummondi	Gratioleae or Mimuleae		
Hemichaena fruiticosa	Mimuleae		
Lancea tibetica	Mimuleae		
Leucocarpus perfoliatus	Mimuleae		
Mazus reptans	Mimuleae		
Mimulus aurantiacus var. *aridus*	Mimuleae	*Schizoplacus*	*Diplacus*
Mimulus clevelandii	Mimuleae	*Schizoplacus*	*Diplacus*
Mimulus brevipes	Mimuleae	*Schizoplacus*	*Eunanus*
Mimulus whitneyi	Mimuleae	*Schizoplacus*	*Eunanus*
Mimulus douglasii	Mimuleae	*Schizoplacus*	*Oenoe*
Mimulus pulchellus	Mimuleae	*Schizoplacus*	*Oenoe*
Mimulus cardinalis	Mimuleae	*Mimulus*	*Erythranthe*
Mimulus lewisii	Mimuleae	*Mimulus*	*Erythranthe*
Mimulus gracilis	Mimuleae	*Mimulus*	*Mimulus*
Mimulus ringens	Mimuleae	*Mimulus*	*Mimulus*
Mimulus uvedaliae	Mimuleae	*Mimulus*	*Mimulus*
Mimulus bicolor	Mimuleae	*Mimulus*	*Paradanthus*
Mimulus floribundus	Mimuleae	*Mimulus*	*Paradanthus*
Mimulus nepalensis	Mimuleae	*Mimulus*	*Paradanthus*
Mimulus depressus	Mimuleae	*Mimulus*	*Simiolus*
Mimulus guttatus	Mimuleae	*Mimulus*	*Simiolus*
Mimulus tilingii	Mimuleae	*Mimulus*	*Simiolus*
Phryma leptostachya var. *leptostachya*	Phrymeae		
Phryma leptostachya var. *asiatica*	Phrymeae		
Peplidium aethocheilum	Gratioleae or Mimuleae		

(Gene Codes Corporation, Ann Arbor, Michigan, USA), from which a consensus sequence was generated.

Analyses—Consensus sequences for *trnL/F*, ITS, and ETS of all taxa were aligned manually using the program Se-Al version 1 (A. Rambaut, University of Oxford, Oxford, United Kingdom). Some regions were too different to be confidently aligned for ITS and ETS sequences, but would be valuable for analyses within subgroups. Regions that were difficult to align with confidence were excluded. Alignments are available at the Botanical Society of America website (http://ajbsupp.botany.org/v89/).

The three DNA regions were analyzed both individually and in combination using the program PAUP* 4.0b3a (Swofford, 1998). The default PAUP settings were used except as noted. Parsimony searches were conducted using heuristic searches with 1000 random sequence addition replicates, to find multiple islands of trees, if present (Maddison, 1991). Gaps were scored as missing data. Support for individual branches was estimated using bootstrap values (Felsenstein, 1985). Bootstrap values were calculated using 1000 full heuristic search replicates.

Maximum likelihood estimates of the phylogeny were also made using PAUP* for the nrDNA, *trnL/F*, and combined data sets. In each case, one of the most parsimonious trees was selected and PAUP* was used to estimate the transition : transversion ratio (ti : tv) (with two rate categories) and the shape parameter for the gamma distribution. Base frequencies were determined using the empirical values. The estimated values were entered as fixed data and were used in a heuristic search. Using these parameters corresponds to the HKY85 model of evolution.

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 different DNA fragments. The following comparisons were made: ITS vs. ETS and *trnL/F* vs. combined ITS and ETS. For each test, 100 replicates were analyzed with an heuristic search, each with ten random sequence addition replicates.

RESULTS

The alignment of the *trnL/F* sequences was straightforward, though numerous short gaps were introduced. We had difficulty amplifying *trnL/F* from *Glossostigma* and were only able to amplify the intron for *Berendtiella*. The total aligned length of the *trnL/F* intron and spacer is 1024 base pairs (bp). The *trnL/F* alignment had 339 variable and 181 parsimony-informative sites. Analyses using maximum parsimony (MP) as the optimality criterion resulted in 700 most-parsimonious trees of 521 steps found on six different islands (consistency index [CI] = 0.785, retention index [RI] = 0.823, rescaled consistency index [RC] = 0.646). For maximum likelihood (ML) analyses of *trnL/F* sequence data, the following parameters were estimated on one of the most parsimonious trees: ti : tv ratio = 1.035, gamma = 0.863. The most likely tree given our data set was one of the 700 MP trees (Fig. 1).

The ITS region is quite variable between distantly related taxa. As a result, only regions that could be aligned unequivocally were used in this analysis, making our phylogenetic estimates using ITS conservative. The total aligned length of the ITS region was 696 bases. The ITS1 had an aligned length of 273 bp in this analysis but only 180 bases could be aligned unambiguously, thus 93 bp were excluded from the analysis (bases 47–133 and 242–247 in the alignment). As expected, the entire 5.8S region, with an aligned length of 165 bases, was aligned unambiguously. The ITS2 had an aligned length of 258 bases of which 200 were unambiguously aligned and used for analysis (bases 455–487, 520–523, 615–627, 652–658, and 665–689 in the alignment were excluded).

Compared to ITS, the approximately 450 bp of the 3′ end of the ETS that we analyzed was relatively easy to amplify and sequence. Complete sequences for both strands required only two sequencing reactions. The aligned ETS sequences were 491 bp in length. Pairwise distances among all sampled taxa ranged from 0.7% to 40.1% for ETS compared to a range of 0% to 27.9% for ITS. The GC content of ETS (56.2%) was similar to that of ITS (59.2%). Seven regions totaling 123 bp

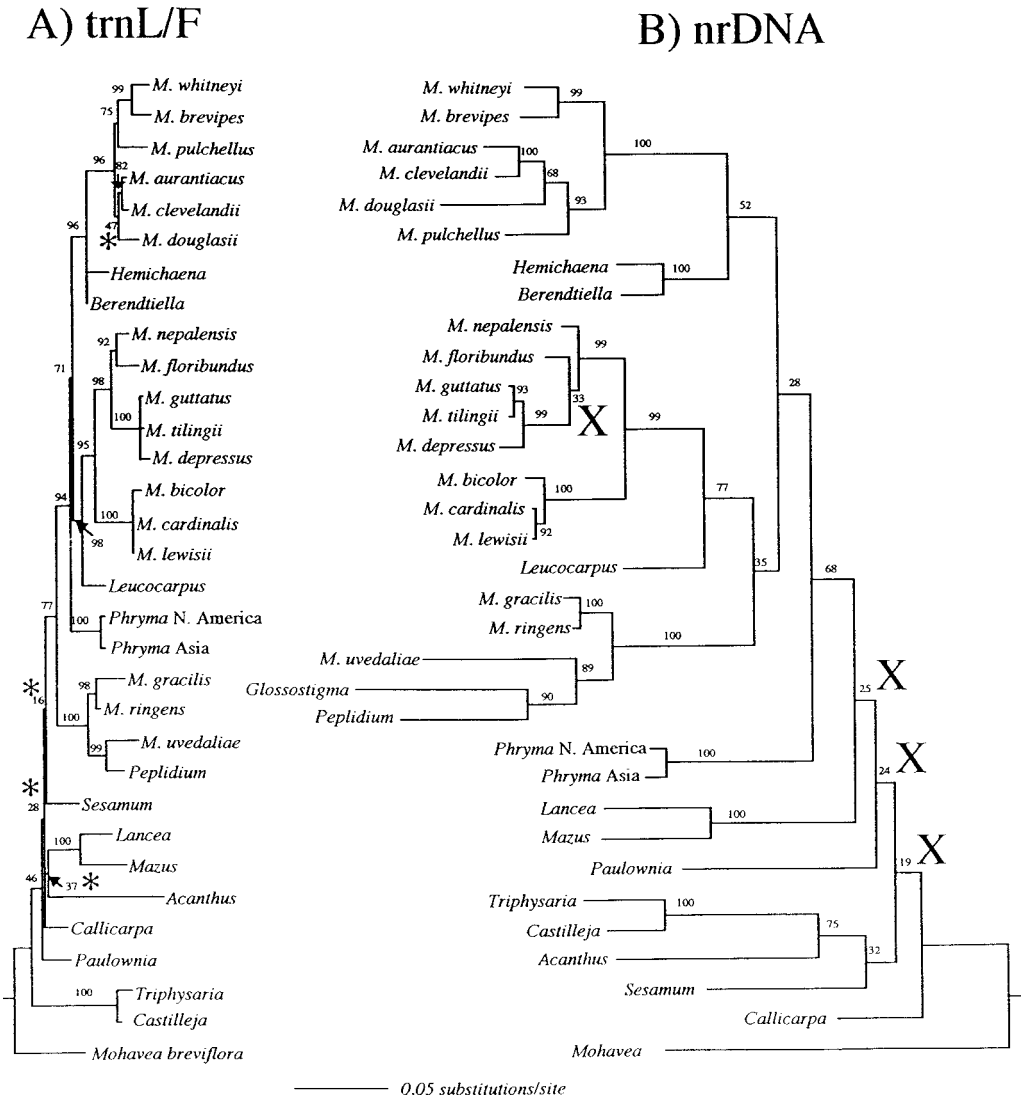

Fig. 1. (A) The maximum likelihood (ML) tree resulting from the analysis of *trnL/F* data; this tree is also one of the 700 most-parsimonious (MP) trees. An asterisk indicates nodes that collapse in the strict consensus of MP trees. (B) The ML tree resulting from the analysis of nrDNA ITS and ETS sequence data. An X on this tree indicates nodes that differ from the MP tree. In both trees, numbers above the branches indicate bootstrap values estimated using parsimony.

could not be aligned confidently and were excluded from the analysis (bases 93–104, 159–200, 263–284, 368–373, 390–398, 439–451, and 470–488 in the alignment).

The ETS and ITS region are closely linked in the rDNA, therefore, we will only present results for analyses of combined rDNA data. Results from the partition homogeneity test (see below) justify our decision. The combined nrDNA data set was 1187 bases in aligned length. After excluding ambiguously aligned sites, 874 bases were used in the analysis, of which 540 were variable and 365 were parsimony informative. Parsimony analyses resulted in one most-parsimonious tree of length 1586 (CI = 0.541, RI = 0.656, RC = 0.355). For the ML analysis of nrDNA sequence data (Fig. 1), the following parameters were estimated on one of the most-parsimonious trees: ti : tv ratio = 2.037, gamma = 0.634. The MP tree differed from the ML tree in the resolution of the deeper nodes and the ML tree resolves *M. floribundus* as sister to *M. nepalensis* while the MP tree resolves *M. nepalensis* as sister to a clade containing *M. floribundus*, *M. guttatus*, *M. tilingii*, and *M. depressus*.

Results of the partition homogeneity test for ITS vs. ETS and ITS/ETS vs. *trnL/F* showed that none of the data sets were significantly different from random pairwise partitions of the data ($P = 0.14$, $P = 0.67$, respectively). Therefore, we combined all three data sets in subsequent analyses.

The combined chloroplast and nrDNA data set was 2211 bases in aligned length. A total of 313 sites were difficult to align and were excluded, resulting in 1898 included characters, of which 879 were variable and 546 were parsimony informative. Parsimony analyses of the combined data resulted in three most-parsimonious trees of length 2117 (CI = 0.599, RI = 0.691, RC = 0.413). For the ML analysis of the combined data, the following parameters were estimated on one of the most parsimonious trees: ti : tv ratio = 1.164, gamma = 0.471. The ML tree is congruent with the strict consensus of the three MP trees and has a parsimony length of 2118.

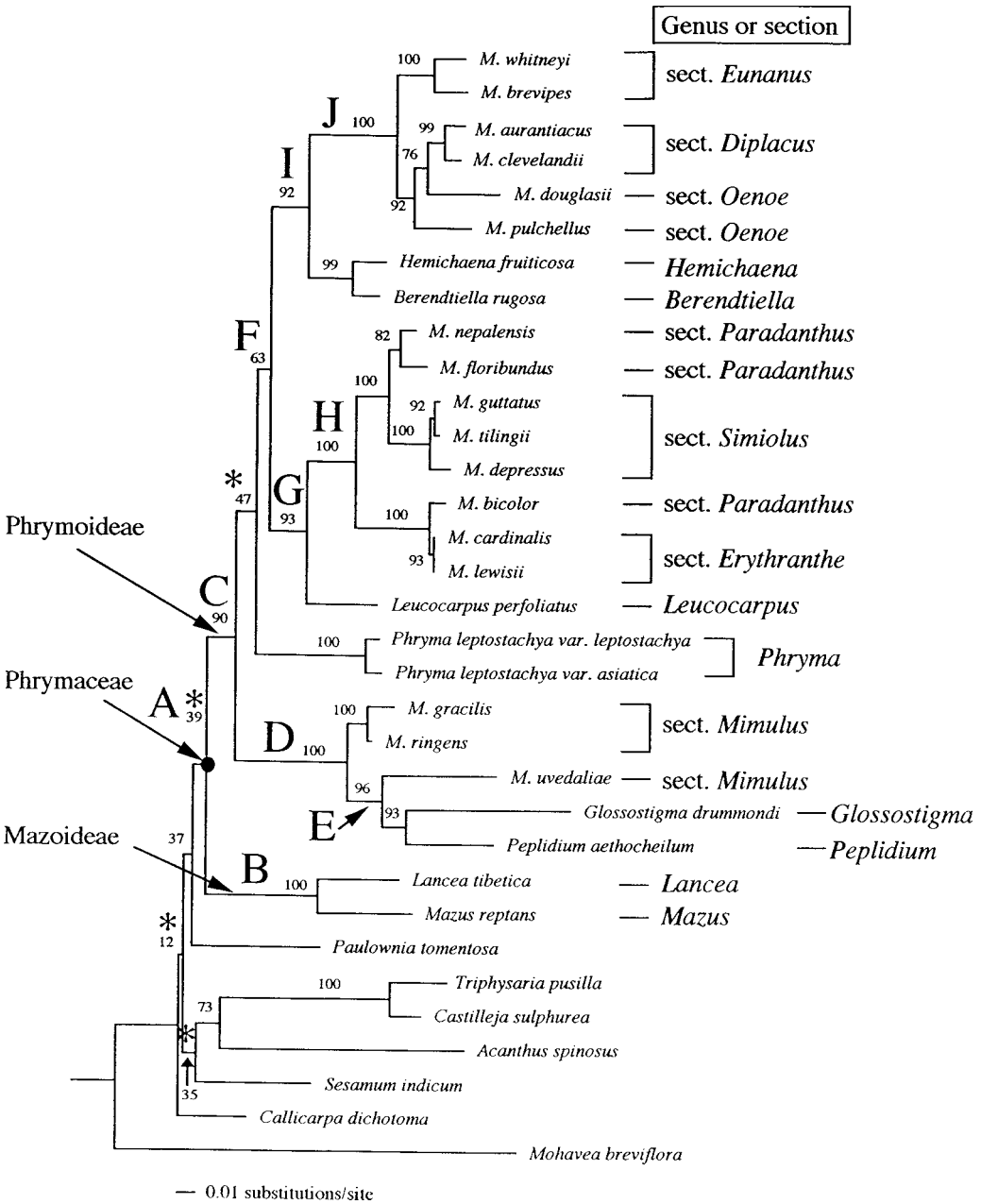

Fig. 2. Maximum likelihood tree inferred using combined data. Numbers above the branches indicate bootstrap values based on parsimony analyses. An asterisk indicates the nodes that collapse in the strict consensus of the three most parsimonious trees. Sampled genera and sections within *Mimulus* are listed to the right of the tree. Capital letters denote groups mentioned in the text.

Figure 1 compares the ML trees resulting from the analysis of chloroplast DNA and nrDNA. The phylogeny estimated using *trnL/F* shows good resolution of deep nodes among genera of Mimuleae but poor resolution among the families in the Lamiales. The phylogeny estimated using nrDNA resolves some of the deeper nodes in the phylogeny differently, though none are well supported. Analyses of the combined data (Fig. 2) resulted in a topology that is congruent with the phylogeny estimated using only chloroplast data, though more resolved, for the relationships of species of *Mimulus*, *Phryma*, *Leucocarpus*, *Hemichaena*, *Berendtiella*, and *Peplidium* with the exception of the placement of *M. pulchellus*. Relationships among and within these genera are identically resolved in the combined ML tree and one of the combined MP trees. The relationships of families in the Lamiales to each other are generally unresolved in all analyses. The major clades identified in our analyses are detailed below.

Clade A (Fig. 2) was recovered in the combined ML analysis, though support was weak (bootstrap [BS] = 39%). Morphological evidence supports our recognition of this clade as the family Phrymaceae. In clade B, *Lancea* and *Mazus* are resolved as sister to each other (BS = 100% all analyses) and is recognized at the subfamily rank as Mazoideae. Clade C is well supported in all analyses (BS = 77% cpDNA, 68% nrDNA, 90% combined) and includes four genera from tribe Mimuleae sensu Argue (1984) (*Mimulus*, *Leucocarpus*, *Hem-*

ichaena, and *Berendtiella*) and *Glossostigma*, *Peplidium*, and *Phryma*. We refer to this clade as the subfamily Phrymoideae. The relationships among Phrymoideae, Mazoideae, and *Paulownia* are unresolved in the strict consensus of the three MP trees.

Within Phrymoideae, relationships among all sampled taxa are fully resolved in the combined analysis, with the exception of one node in the MP analyses, and most clades are well supported. Of the 20 resolved nodes in the MP analyses, only one has a bootstrap value <76%, and only three have bootstrap values <92%. Clade D (BS = 100% all analyses) contains members of *Mimulus* sect. *Mimulus* (sect. *Eumimulus* sensu Grant [1924]), including the type species, *M. ringens*, and the Australian genera *Glossostigma* and *Peplidium*. Derived from within clade D is clade E (BS = 96%), containing *Glossostigma*, *Peplidium*, and *M. uvedaliae*, which also is Australian in distribution. A sister relationship between sampled members of *Glossostigma* (which was not sampled for *trnL/F*) and *Peplidium* was recovered in analysis of nrDNA (BS = 90%).

All *Mimulus* in western North American plus the genera *Berendtiella*, *Hemichaena*, and *Leucocarpus* form clade F (BS = 63%). *Phryma* from eastern North America and from eastern China form a clade (BS = 100%). *Phryma* is sister to clade F in the ML analysis of combined data and in two of the three MP trees. In one MP tree, *Phryma* is sister to the entire Phrymoideae. Clade G (BS = 93%) contains *Leucocarpus* and its sister clade H (BS = 100%), which contains *Mimulus* sects. *Simiolus*, *Erythranthe*, and *Paradanthus*. Within clade H are clades that correspond to sect. *Erythranthe* (*M. cardinalis* and *M. lewisii* [BS = 93%]) and sect. *Simiolus* (*M. guttatus*, *M. tillingii*, and *M. depressus* [BS = 100%]). Within *Simiolus*, the two species from western North America form a clade (BS = 92%) that is sister to the species from Chile (*M. depressus*). Section *Paradanthus* is paraphyletic.

Berendtiella and *Hemichaena*, together with all members of *Mimulus* subgenus *Schizoplacus* form clade I (BS = 92%). All sampled species in *Mimulus* subg. *Schizoplacus* are monophyletic (BS = 100%) and together comprise clade J that is sister to a clade comprised of *Hemichaena* and *Berendtiella* (BS = 99%). Within clade J are clades that correspond to sect. *Eunanus* (*M. whitneyi* and *M. brevipes* [BS = 100%]) and sect. *Diplacus* (*M. aurantiacus* and *M. clevelandii* [BS = 99%]). The latter plus two species of *Mimulus* sect. *Oenoe* form a clade (BS = 92%). *Diplacus* appears to be derived from within a paraphyletic *Oenoe*.

DISCUSSION

Estimates of phylogeny from chloroplast and nuclear data—Data from the chloroplast and the nucleus are largely congruent with respect to phylogenetic inference and, in combination, provide a powerful data set for resolving relationships within the group. The nrDNA and cpDNA data are complementary in that the nrDNA provides better resolution near the tips of the tree whereas the cpDNA resulted in more strongly supported nodes at deeper levels within the Phrymoideae. This result was expected based on the results of ITS analyses of other closely related families in Lamiales (McDade et al., 2000). Neither source of data provided resolution of the relationships of Phrymaceae to other families in the Lamiales. The chloroplast data were less homoplastic than the nuclear data (*trnL/F* CI = 0.776 vs. nrDNA CI = 0.518) for analyses of all taxa. The combined analysis reflects the strongly supported components of the individual data sets and results in a well-supported estimate of phylogeny in *Mimulus* and related genera in tribe Mimuleae from which significant systematic and evolutionary conclusions can be drawn.

Systematic conclusions—(1) *Mimulus* and the genera derived from within it form a strongly supported clade referred to as subfamily Phrymoideae (Fig. 2). This is not nested in any clade traditionally considered to have the rank of family in this study. Similarly, the recent study of Olmstead et al. (2001) found that *Mimulus* was not associated with any of the named clades of Lamiales. Phrymoideae has a wide geographic range and contains approximately 160 species that are divergent morphologically and ecologically.

(2) There is weak support for a more inclusive clade, Phrymaceae, in which Phrymoideae and Mazoideae are sister groups. This relationship is recovered in the ML analysis of the combined data, in two of the three MP trees, and in 39% of the parsimony bootstrap data sets. Given that *Mazus* and *Lancea* share a set of morphological characters with species in Phrymoideae, we tentatively assign these genera to Phrymaceae with the caveat that further research is needed to confirm their sister relationship to Phrymoideae. Therefore, we propose that the clade recovered in this analysis that contains all the representatives of *Mimulus*, as well as the genera *Phryma*, *Glossostigma*, *Peplidium*, *Leucocarpus*, *Berendtiella*, and *Hemichaena*, *Mazus*, and *Lancea* be recognized at the rank of family; Phrymaceae (Schauer, 1847) has priority. A distinct advantage in recognizing *Lancea* and *Mazus* within the Phrymaceae is that synapomorphies can be diagnosed for Phrymaceae, as will be discussed below in the section on morphological evolution. The scope of this newly defined Phrymaceae is very different from its historical antecedents. Previously, the family was monotypic and limited in geographic range to eastern North America and eastern China. In our circumscription, Phrymaceae encompasses approximately 190 species that are distributed worldwide.

(3) Relationships of Phrymaceae to other families in the Lamiales are generally unresolved. A similar lack of resolution has been encountered in other molecular analyses of the Lamiales (Olmstead et al., 2001; B. Oxelman, Uppsala University, unpublished manuscript). Sampling in this study was designed to include groups putatively closely related to *Mimulus* and tribe Mimuleae, based on other molecular studies of Lamiales. Given a limited sampling of these groups, *Paulownia* has the strongest support (albeit weak; BS = 37%) as sister to Phrymaceae. This relationship is recovered in the ML analysis and in two of the three MP trees. The third MP tree resolves *Paulownia* as sister to Phrymoideae and they, in turn, are sister to Mazoideae. If this latter relationship turns out to be correct, *Paulownia* would simply be included within Phrymaceae. At present, we assign *Paulownia* to the monogeneric family Paulowniaceae.

(4) The genus *Mimulus* is not monophyletic. *Glossostigma*, *Peplidium*, *Phryma*, *Leucocarpus*, *Hemichaena*, and *Berendtiella* are derived from within *Mimulus*. Though it was not sampled, morphological evidence strongly implies that the Australian genus *Elacholoma* is also derived from within *Mimulus* (Barker, 1982). The monophyly of *Mimulus* was not found in analyses of any of the individual data sets or in any combination of the data sets. In the combined data set, trees that are constrained to make *Mimulus* monophyletic are 94

steps longer. The nonmonophyly of *Mimulus* is not surprising, given that no clear synapomorphy for the group has been identified. In her monograph of *Mimulus*, Grant (1924) suggested that calyx characters best diagnosed the genus. Calyx characters within *Mimulus*, however, cannot be described in any way that would consistently differentiate *Mimulus* from other genera in tribe Mimuleae.

(5) It is clear that generic boundaries within Phrymaceae require extensive redefinition in order for genera to be monophyletic. Options for generic revisions include: (i) retain the traditional *Mimulus* sensu lato (s.l.) as one genus and reassign species of the six other genera to *Mimulus* or (ii) break up the traditional genus *Mimulus*. The advantages of option i are that fewer species in Phrymaceae would require a name change and all of the intensely studied species in western North America would retain the name with which they are currently identified. In this scenario, Phrymoideae would be synonymous with *Mimulus*. The primary disadvantages of option i are that at least six genera would require name changes and that all the diversity contained in Phrymoideae would be assigned to one genus. The latter situation is only a problem, however, if one assumes that different ranks within a hierarchical classification have implications with regards to amounts of diversity. Option ii would result in more genera in Phrymaceae than option i but would require 105 to 185 name changes, depending on where generic boundaries are drawn. Redrawing generic boundaries within Phrymaceae and in-depth discussions of the implications of using traditional and phylogenetic nomenclatural systems (deQueiroz and Gauthier, 1992; Cantino et al., 1999) are beyond the scope of this paper, but will be the subject of a future publication.

(6) Some subgeneric and sectional relationships are apparent. Subgenus *Schizoplacus* is shown to be monophyletic (BS = 100%) whereas subgenus *Mimulus* is not. Preliminary evidence exists for the monophyly of sects. *Simiolus* (BS = 100%), *Erythranthe* (BS = 93%), *Diplacus* (BS = 99%), and *Eunanus* (BS = 100%). Section *Mimulus* is not monophyletic, because *Glossostigma* and *Peplidium* are derived from within it, nor is section *Paradanthus*. In defining sect. *Paradanthus*, Grant (1924) admitted that the species within it were probably not closely related. The nonmonophyly of this section was also postulated from pollen data (Argue, 1980). *Mimulus bicolor* (sect. *Paradanthus*) is closely related to species in *Erythranthe*, whereas other species in *Paradanthus* are more closely related to sect. *Simiolus*. Section *Oenoe* may also not be monophyletic. More thorough analyses of relationships within *Mimulus* in western North America and Australia are in preparation.

Morphological evolution—Species within the redefined Phrymaceae are diverse in both life history and morphological traits. This clade includes species with habits ranging from annuals, attaining a final plant height of a few centimeters, to perennials, some of which are woody and attain a height of 4 m. Variation for reproductive mechanisms also exists, including species that have outcrossing, mixed-mating, selfing, and asexual breeding systems. At least five species in Phrymaceae, *M. congdonii*, *M. douglasii* (Thompson, 1993), *M. nasutus* (Diaz and Macnair, 1998), *M. pictus* (Thompson, 1997), and *Glossostigma cleistanthum* (Barker, 1982), have some populations that are cleistogamous. Within Phrymaceae, insects pollinate flowers of most of the species, but hummingbird pollination also exists (*M. cardinalis* [Bradshaw et al., 1998] and

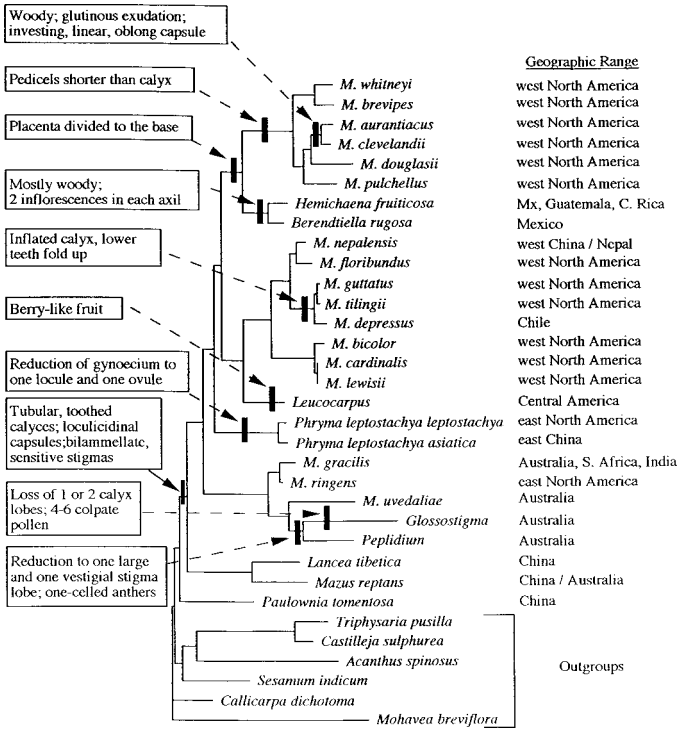

Fig. 3. Inferred morphological changes in Phrymaceae using the ML estimate of the phylogeny from the combined data.

M. flemingii [Stebbins, 1989]). Many species have corollas with bilateral symmetry, but flowers of other species are radially symmetric. Barker (1982) notes that radially symmetrical corollas seem to be associated with a prostrate habit in Australian species of *Mimulus* and *Peplidium*. The most common fruit type in Phrymaceae is a readily dehiscent capsule containing numerous seeds, but exceptions exist. *Phryma leptostachya* has an achene (Whipple, 1972). *Leucocarpus perfoliatus* has a baccate, indehiscent fruit (Burtt, 1965) described as a white berry, with thin skin and with most of the substance of the fruit derived from the fleshy placenta. Many species of *Peplidium* possess capsules that are thick-walled and open only after the plant has senesced. Grant (1924) described a similar capsule in some species of *Mimulus* sect. *Oenoe*. The phylogenetic distance between *Mimulus* sect. *Oenoe* and *Peplidium* (Fig. 2) demonstrates that this character evolved convergently in taxa from two different continents, possibly in response to common environmental conditions. Species in Phrymaceae also show variation in habitats, inhabiting sites as varied as desert, riparian, and alpine environments. Some species extend over a large geographic range and have developed many different races (e.g., *M. glabratus*) whereas others are local endemics and several are rare (e.g., *M. exiguus*).

It is of great practical importance to provide morphological synapomorphies for described clades. Synapomorphies for some of the recovered clades are described below, along with evidence for or against previous systematic and morphological hypotheses. A summary of the major morphological shifts in the family is presented in Fig. 3.

Phrymaceae—Phrymaceae have the following synapomorphies: (1) tubular, toothed calyces, (2) loculicidal capsules, and (3) bilamellate stigmas that are receptive only on the inner

surface and close together upon contact. This combination of characters can be used to segregate members of Phrymaceae from other families in the Lamiales. However, Phrymaceae are diverse morphologically and some clades and individual species have derived character states that may make morphological assessment difficult. Stigmatic lobes are much reduced in some species in association with reduced floral displays (e.g., *Phryma leptostachya* and *M. nasutus*) and other suites of traits typically associated with a highly selfing mating system making the assessment of stigma sensitivity to touch difficult. *Glossostigma* also has a derived stigma structure (described below).

Mimulus subgenus Schizoplacus—Grant (1924) diagnosed subgenus *Schizoplacus* primarily on the basis of having a placenta that is divided to the base. Other traits associated with *Schizoplacus* were relatively short pedicels (usually shorter than the calyx) and glandular pubescent styles. Sister taxa *Hemichaena* and *Berendtiella* have united placentae thus making divided placentae a synapomorphy for *Schizoplacus*. *Berendtiella* and *Hemichaena* have pedicels that are shorter than the calyx and glandular pubescent styles, making these characters synapomorphies for the more inclusive clade.

Mimulus section Mimulus—The characters that Grant (1924) used to diagnose sect. *Mimulus* (*Eumimulus* sensu Grant [1924]) were tubular calyces with equal teeth and a distinctly bilabiate, blue corolla with glabrous palatine ridges. Presumably, the description of tubular calyces was meant to distinguish the calyces in sect. *Mimulus* from those in sect. *Simiolus*, which are strongly inflated and sagitally compressed, and sects. *Paradanthus* and *Erythranthe*, which have sharp, definite angles and flat sides. However, according to our results, these characters are not consistent within the clade containing sect. *Mimulus*. The Australian genera *Glossostigma* and *Peplidium* are clearly derived from within sect. *Mimulus* and do not have equal calyx teeth, many have radially symmetric flowers, many others have flowers that are not blue, and some have sharply angled calyx lobes. Overall, the distribution of morphological characters in this clade needs to be reexamined in light of the phylogenetic evidence. At present, no clear synapomorphies exist for the clade containing *Mimulus* sect. *Mimulus*.

Glossostigma/Peplidium—Barker (1982) described two derived morphological characters for these genera: one-celled anthers and the presence of one large and one vestigial stigmatic lobe. In *Glossostigma* and *Peplidium*, the large stigmatic flap covers the corolla mouth and, upon being touched, is triggered back against the upper corolla lip. This stigmatic movement is apparently homologous to and derived from the stigmatic condition in the rest of the Phrymaceae in which two equal or nearly equal stigma flaps, receptive on the inner surface only, close upon touch (Fetscher and Kohn, 1999).

Phyrma—The monotypic genus *Phryma* has been difficult to place taxonomically due to its having a pseudomonomerous gynoecium that develops into an achene. The results presented here indicate that *Phryma* is derived from within the traditionally recognized genus *Mimulus*. In comparison to other species in Phrymaceae, *Phryma* shares some floral characteristics, but many of its reproductive characters are derived. All other taxa in the Phrymaceae are bicarpellate and have numerous seeds, thus the enigmatic gynoecium and fruit of *Phryma* is derived. Whipple (1972) described the calyx of *Phryma* as persistent, zygomorphic, and as having five ridges with three hooked adaxial lobes and two short subulate abaxial lobes. The three elongated and hooked adaxial corolla lobes can be interpreted as being derived from the ancestral condition of relatively short, uncurved adaxial lobes. It is possible that this morphological switch is an adaptation to epizoochorous dispersal, in that these hooked upper lobes allow the fruit to become attached to animal fur (Holm, 1913). The flowers of *Phryma* have two stigmatic surfaces at the tip of the style, but, in general, flowers are much reduced, which makes determination of stigmatic characters difficult. *Phryma* is also characterized by the reflexed movement of the mature calyx and fruit (Whipple, 1972). While in flower, the calyx is perpendicular to the stem, while in fruit the calyx points abaxially and is parallel to the stem. Its common name ''lopseed'' reflects this fruit orientation.

Diplacus—The following characters have been used to diagnose *Diplacus*: (1) plants are shrubs, semi-shrubs, or perennial from a woody caudex; (2) glutinous exudation from the leaves; (3) prismatic calyx; and (4) investing, linear, oblong capsule (Grant, 1924). This combination of characters separates *Diplacus* from other groups in Phrymaceae.

Hemichaena-Berendtiella—In an analysis of morphological characteristics of *Hemichaena*, *Berendtiella*, and *Leucocarpus*, Thieret (1972) suggested that *Hemichaena* and *Berendtiella* could be distinguished from other closely related genera by their inflorescences of bracteolate cymes. Species in this clade are woody, as are members of the *Diplacus* clade, suggesting the convergent evolution of wood in these two groups (Fig. 3). Additionally, species in both *Diplacus* and the *Hemichaena-Berendtiella* clade have leaves that are revolute at their margins, a character that is possibly a convergent adaptation to the dry habitats in which plants of both groups live. Thieret (1972) postulated that *Leucocarpus* and *Hemichaena* were closely related due to their possession of reticulate seeds and distinctive calyces and stigmas. The estimated phylogeny (Fig. 2), however, shows that *Leucocarpus* and *Hemichaena* are not closely related. The calyx and stigma characters are sympleisiomorphies.

A final observation made by Thieret (1972) on the *Hemichaena-Berendtiella* clade is the curious presence of two flowers in each axil that he called superposed inflorescences. The presence of two supernumerary buds in the axil of each leaf has also been reported in *M. guttatus* and *M. gemmiparus* (Moody, Diggle, and Steingraeber, 1999). In each of these species, the distal bud ultimately becomes a flower or a lateral branch. In *M. guttatus*, the proximal bud remains inactive for the entire life of the plant. In *M. gemmiparus*, the proximal bud becomes a brood bulbil that functions as an asexual propagule, which is the primary means of propagation of this species (Beardsley, 1997). Therefore, among North American Phrymaceae, supernumerary buds can be seen to take on at least three different forms: a flower in *Hemichaena-Berendtiella*, a brood bulbil in *M. gemmiparus*, and dormant in *M. guttatus*.

Biogeography—Within Phrymoideae are two clades that represent distinct radiations and document two centers of diversity. The first center of diversity is in western North Amer-

ica and is well recognized and often studied. From within this radiation of species in western North America, several species have colonized other continents, possibly through long-distance dispersal. *Mimulus depressus* (Chile) is a member of sect. *Simiolus*, indicating a relatively recent establishment of *Mimulus* in South America. The presence of a suite of morphological traits makes it highly probable that most species of *Mimulus* in Chile are closely related and reflect one colonization event. Pollen (Argue, 1981) and other morphological data (von Bohlen, 1995b) indicate that *M. crinitus* and *M. bridgesii* are not closely related to other Chilean *Mimulus* and reflect at least one additional colonization event. *Mimulus nepalensis* (Tibet and China) is derived from within a clade of taxa that are found primarily in California and the Pacific Northwest. In addition, morphological evidence (Grant, 1924) and preliminary sequence data (P. M. Beardsley, unpublished data) suggest that *M. sessilifolius* represents a second, distinct, relatively recent establishment of *Mimulus* in Asia.

The greatest number of species in North America occurs in California, with declining numbers of species to the north, extending to southern Alaska. Fewer species occur in the Great Basin, Rocky Mountains, and the desert Southwest. Some species not sampled in this study in sections *Erythranthe*, *Simiolus*, and *Paradanthus* are also found in Mexico. Our data suggest that these species were derived relatively recently from ancestors in California.

The *Hemichaena-Berendtiella* clade is found primarily in central and southern Mexico, and one species, *Hemichaena fruiticosa*, is native to southern Mexico, Guatemala, and Costa Rica (one of the two species in the family with a tropical distribution). *Hemichaena fruiticosa* is found in diverse habitats, both disturbed and undisturbed, in the mountains from 900 to 3500 m (Thieret, 1972). The second species in Phrymaceae found in Central America is *Leucocarpus perfoliatus*, which grows along stream banks in forests from 1350 to 2000 m (Pennell, 1920). *Leucocarpus* is sister to a large clade (approximately 60 species) that radiated extensively in western North America, indicating that some combination of higher rates of speciation or lower rates of extinction in temperate habitats than in tropical habitats exists in this clade.

Phrymaceae have undergone a second radiation in Australia. All Australian taxa within *Mimulus*, *Glossostigma*, and *Peplidium*, together comprising approximately 30 species, fall within one clade. The geographic division between the Australian species and the rest of the taxa in Phrymoideae, which are primarily North American, is certainly of ancient origin. The reconstructed ancestral distribution of the common ancestor of Phrymoideae is unresolved.

Populations of *Phryma leptostachya* exist in eastern North America (var. *leptostachya*) and eastern China and Japan (var. *asiatica*). Similar to other studies (Lee et al., 1996; Xiang et al., 2000), we recovered substantial divergence in the sampled DNA sequences between the two varieties. Using a molecular clock based on divergence in *rbcL* sequences, Xiang et al. (2000) estimated the time of divergence between the two varieties at 5.45 ± 2.47 million years. Hara (1962) demonstrated limited morphological divergence between the two varieties. Differences were limited to the shape, size, and pubescence of leaves. This lack of differentiation is perhaps explained by *Phryma*'s greatly reduced floral display that offers limited opportunities for morphological change. Closely related groups of species in the newly defined Phrymaceae often differ morphologically in floral characters, while retaining pleisiomorphic vegetative characters.

The results of this study begin to clarify patterns of relationship in *Mimulus*, tribe Mimuleae, and other closely related genera. In light of this molecular evidence, we propose the recognition of a dramatically expanded Phrymaceae containing many species that have become model systems for the study of evolutionary processes. Future phylogenetic studies in Phrymaceae will continue to inform the systematics of the group and will serve as a useful starting point for further comparative analyses.

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