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EVOLUTION OF BIOGEOGRAPHIC DISJUNCTION BETWEEN EASTERN ASIA AND EASTERN NORTH AMERICA IN *PHRYMA* (PHRYMACEAE)[1]

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This study examines molecular and morphological differentiation in *Phryma* L., which has only one species with a well-known classic intercontinental disjunct distribution between eastern Asia (EA) and eastern North America (ENA). Phylogenetic analysis of nuclear ribosomal ITS and chloroplast *rps16* and *trnL-F* sequences revealed two highly distinct clades corresponding to EA and ENA. The divergence time between the intercontinental populations was estimated to be 3.68 ± 2.25 to 5.23 ± 1.37 million years ago (mya) based on combined chloroplast data using Bayesian and penalized likelihood methods. Phylogeographic and dispersal-vicariance (DIVA) analysis suggest a North American origin of *Phryma* and its migration into EA via the Bering land bridge. Multivariate analysis based on 23 quantitative morphological characters detected no geographic groups at the intercontinental level. The intercontinental populations of *Phryma* thus show distinct molecular divergence with little morphological differentiation. The discordance of the molecular and morphological patterns may be explained by morphological stasis due to ecological similarity in both continents. The divergence of *Phryma* from its close relatives in the Phrymaceae was estimated to be at least 32.32 ± 4.46 to 49.35 ± 3.18 mya.

Key words: biogeography; intercontinental disjunction; eastern Asia; eastern North America; morphological stasis; *Phryma*; Phrymaceae; phylogeography.

Patterns of intercontinental disjunction in the northern hemisphere usually involve four well-known areas, eastern Asia (EA), eastern North America (ENA), western North America (WNA), and Europe (Milne and Abbott, 2002; Donoghue and Smith, 2004). The EA–ENA disjunction is a well-known classic biogeographic pattern (Li, 1952) that has received considerable attention in the last decade (Xiang et al., 1996, 1998, 2000; Wen, 1999, 2001; Manos and Donoghue, 2001). Morphological similarity has been observed in many disjunct species, and some were originally described as a single intercontinental species with distributions in both EA and ENA (Halenius, 1750; Li, 1952). Parks and Wendel (1990) reported a high level of allozyme and cpDNA divergence in the morphologically similar *Liriodendron chinense* (Hemsl.) Sargent from EA and *L. tulipifera* L. from ENA. Molecular and fossil data suggest a divergence time for the two species of 10–16 million years ago (mya). This long-term morphological stasis observed in *Liriodendron* was subsequently proposed for the EA and ENA species of *Aralia* sect. *Dimorphanthus* (Wen, 2000), *Liquidambar* (Hoey and Parks 1991; Shi et al., 1998), *Magnolia* sect. *Rytidospermum* (Qiu et al., 1995a, b), and *Osmorhiza* (Wen et al., 2002). Morphologically similar species from these two areas in *Aralia*, *Magnolia*, and *Osmorhiza* form paraphyletic or polyphyletic groups, suggesting that the morphological similarities in these groups may be attributable to symplesiomorphy or convergence, respectively (Wen, 1999, 2001). Most of the studies on species with disjunct distributions in these two areas have focused on estimating phylogenetic relationships among EA and ENA taxa and estimating divergence times, yet few have rigorously analyzed patterns of morphological variation among the disjunct taxa.

Phryma L. is distinctive in having a pseudomonomerous gynoecium (two-carpellate with one carpel reduced developmentally). It has a synsepalous calyx with three upper lobes subulate and hooked and a one-seeded achene enclosed in an accrescent calyx (Thieret, 1972; Chadwell et al., 1992). The familial placement of *Phryma* has been controversial, having been placed in Scrophulariaceae, Acanthaceae, Lamiaceae, Verbenaceae, and its own monotypic family Phrymaceae (Holm, 1913; Engler and Prantl, 1936; Rao, 1952; Hutchinson, 1959; Thieret, 1972; Whipple, 1972; Dahlgren, 1980; Cronquist, 1981; Takhtajan, 1987; Lu, 1990; Chadwell et al., 1992). Phrymaceae has recently been recircumscribed to include six genera previously placed in tribe Mimuleae (*Berendtiella*, *Hemichaena*, *Lancea*, *Leuco-*

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carpus, *Mazus*, and *Mimulus*), *Phryma*, *Elacholoma*, *Glossostigma*, *Microcarpaea*, and *Peplidium* based on chloroplast *trnL-F* and nuclear ribosomal ITS and ETS sequence data (Beardsley and Olmstead, 2002; Beardsley and Barker, 2005). Oxelman et al. (2005), however, questioned the placement of *Lancea* and *Mazus* in Phrymaceae sensu Beardsley and Olmstead (2002).

Phryma is one of a few monotypic and taxonomically isolated genera with a high level of morphological similarity in intercontinental populations (Li, 1952; Hara, 1969; Thieret, 1972; Whipple, 1972; Ramana et al., 1983). In flowering plants, only *Phryma leptostachya* L. and *Toxicodendron radicans* (L.) Kuntze include disjunct intercontinental populations in both EA and ENA that are considered to be varieties or subspecies of the same species. Hara (1966) pointed out that disjunct populations of *Phryma* were identical in most morphological features, cytology, and ecological habitats, such as flowers erect in bud but later spreading or becoming deflexed, equal numbers of chromosomes ($2n = 28$; Löve and Löve, 1982; Rudyka, 1995; Sun et al., 1996), and similar habitats of deciduous or mixed forests. Plants from the two different regions differ slightly in leaf size, shape of upper lip of the corolla, and length of the upper spinulose calyx-lobes (Hara, 1962, 1966, 1969; Li, 2000). The EA and ENA populations were thus generally treated as two varieties (Hara, 1966; Thieret, 1972) or subspecies (Kitamura and Miurata, 1957; Li, 2000) of a single intercontinental disjunct species. Recent molecular studies (Lee et al., 1996; Xiang et al., 2000; Beardsley and Olmstead, 2002) detected substantial molecular divergence between the two intercontinental varieties. However, in all previous studies, either only one population was sampled from each continent or the focus was on its systematic position in the Lamiales (Wagstaff and Olmstead, 1997). Comparisons based on a broad sampling scheme from both continents are needed to better understand the phylogeographic structure of *Phryma*. Furthermore, the morphological differentiation between EA and ENA populations has never been examined with quantitative measurements of multiple morphological characters.

Two previous studies estimated the divergence times for the disjunct *Phryma* varieties using a general molecular clock. Lee et al. (1996) reported the divergence time to be over 20 mya using allozymes data and 12.35 mya using ITS sequences. Xiang et al. (2000) estimated the divergence times for several disjunct taxon pairs with *rbcL* sequence data and a molecular clock calibrated with *Cornus* fossils. They estimated that the two varieties of *Phryma* diverged about 5.85 ± 2.66 mya. It is necessary to test the previous estimates using the newly developed methods for estimating divergence times within a phylogenetic framework of *Phryma* and its close relatives. We herein employ both Bayesian dating (Thorne et al., 1998; Thorne and Kishino, 2002) and the penalized likelihood (Sanderson, 2002, 2003) approaches to estimate the timing of the intercontinental disjunction in *Phryma*.

The objectives of this study are to (1) assess molecular divergence and estimate the divergence time between disjunct populations of *Phryma*, (2) document the morphological similarity and differentiation of intercontinental populations, (3) examine the phylogeographic structure of *Phryma* in both continents, and (4) reconstruct the biogeographic history of *Phryma* between EA and ENA.

MATERIALS AND METHODS

Molecular analysis—Sequences from 22 accessions of *Phryma* and three related taxa were used in the study (Fig. 1; Appendix 1). Genomic DNA was extracted from 15 mg of dried leaf material using the modified CTAB method of Doyle and Doyle (1987). The nuclear ribosomal ITS and the chloroplast *trnL-F* regions (including the *trnL* intron and the *trnL-trnF* spacer) and the *rps16* intron were used, because a large number of sequences were already available for the Lamiales in GenBank to enable our biogeographic analysis in the phylogenetic framework of the Lamiales. The ITS and *trnL-F* regions were amplified and sequenced following Beardsley and Olmstead (2002), and the *rps16* intron sequences were obtained according to the protocol in Nie et al. (2005). Sequences were aligned with ClustalX, version 1.83 (PC version, Thompson et al., 1997), followed by manual adjustments.

Phylogenetic analyses were performed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (Rannala and Yang, 1996). *Mimulus aurantiacus* and *Lancea tibetica* of Phrymaceae were used as outgroups because they are close relatives of *Phryma* in the recircumscribed Phrymaceae (Beardsley and Olmstead, 2002). Because of the relatively small number of terminals included in this analysis, the branch and bound search algorithm was used with PAUP* version 4.0b10 (Swofford, 2003). Gaps were scored as missing data. Bootstrap values (BV) are based on 1000 replicates using parsimony and the branch and bound search algorithm (Felsenstein, 1985). The appropriate model of DNA substitution for the maximum likelihood analysis was determined using Modeltest, version 3.6 (Posada and Buckley, 2004). Once the best-fit model was determined, maximum likelihood searches were performed for the data set with a heuristic research, each with 10 random sequence additions. Bayesian inference was conducted using MrBayes, version 3.0 (Huelsenbeck and Ronquist, 2001) with the model as estimated previously. The MCMC algorithm was run for 2 000 000 generations with four incrementally heated chains, starting from random trees and sampling one of every 100 generations. The first 2000 to 5000 trees were discarded as burn-in, depending on when chains appeared to have become stationary, and the remaining trees were used to construct Bayesian consensus trees. Internodes with posterior probabilities $\geq 95\%$ in the consensus trees were considered statistically significant.

The incongruence length difference (ILD) test (Farris et al., 1994, as implemented in PAUP*) of ITS vs. combined chloroplast *rps16* and *trnL-F* regions was used to assess potential conflicts between the phylogenetic signal from different genomes. For each test, 100 replicates were analyzed with heuristic search, each with 10 random sequence additions.

Biogeographic analysis—We used the ML tree generated from combined *rps16* and *trnL-F* data to estimate the divergence time of *Phryma* between EA and ENA with 41 taxa sampled from the Lamiales. Sequences of 36 species were obtained from GenBank (Appendix 2). We sequenced the *rps16* intron and the *trnL-F* region for *Catalpa fargesii* Sweet, *Chilopsis linearis* Bur., and *Macrocatalpa* of the Bignoniaceae because only one species of *Catalpa* was previously sequenced and the additional sampling enabled us to use *Catalpa* fossils as alternative calibration point for estimating the divergence times. A few taxa were coded with missing data in the *rps16* intron region because fewer sequences of the region were available for the Lamiales. Phrymaceae were well sampled with diverse representatives of *Mimulus* as well as other members of the family including *Hemichaena*, *Berendtiella*, *Leucoparus*, *Peplidium*, *Lancea*, and *Mazus* based on the phylogeny in Beardsley and Olmstead (2002).

A likelihood ratio test (Felsenstein, 1988) was carried out to test whether the two chloroplast markers evolved in a clock-like fashion. This test resulted in $P < 0.05$, suggesting that rate constancy in this data set was not supported. We therefore used both Bayesian dating (Thorne et al., 1998; Thorne and Kishino, 2002) and penalized likelihood (PL, Sanderson, 2002) to estimate divergence times.

Bayesian dating is based on the assumption that simultaneous analysis of several gene loci with multiple calibrations will overcome not only the often weak signal in single data sets but also violations of the clock in each of the individual partitions (Thorne et al., 1998; Thorne and Kishino, 2002; Yang and Yoder, 2003). It uses a probabilistic model to describe the change in evolutionary rate over time and uses the MCMC procedure to derive the posterior distribution of rates and time. It allows multiple calibration windows and provides direct standard deviations and credibility intervals for estimated divergence times and substitution rates. The procedure we followed is divided into three different steps and programs and is described in more detail in a step-by-step manual available at website http://www.plant.ch/software.html. In the

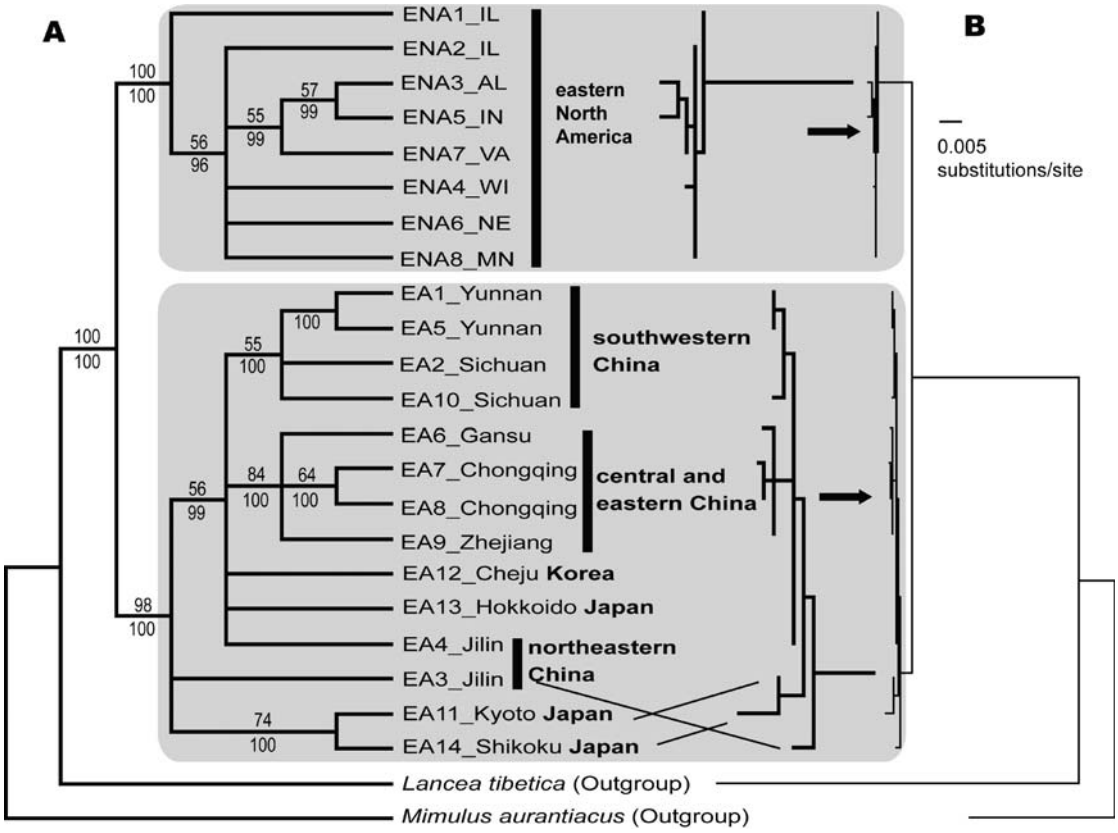

Fig. 1. Strict consensus tree (A) and maximum likelihood tree (B) resulting from combined ITS, *rps16*, and *trnL-F* data (tree length = 396 steps, CI = 0.97, RI = 0.96, and RC = 0.92). The bootstrap values in 1000 replicates are shown above the lines and the Bayesian Markov chain Monte Carlo (MCMC) posterior probabilities below the lines.

first step, we used the "baseml" program in the PAML package, version 3.14 (Yang, 1997), and the F84 + G model (Kishino and Hasegawa, 1989) to estimate base frequencies, transition/transversion rate kappa, and the alpha shape parameter (five categories of rates). Then, by using these parameters, we estimated the maximum likelihood of the branch lengths of the rooted evolutionary tree together with a variance–covariance matrix of the branch length estimates using the program Estbranches (Thorne et al., 1998). The maximum likelihood scores obtained in baseml and Estbranches were then compared to determine if both approaches were able to optimize the likelihood. The program Multidivtime (Thorne and Kishino, 2002) was used to approximate the posterior distributions of substitution rates and divergence times by using a multivariate normal distribution of estimated branch lengths and by running a MCMC procedure following data-dependent settings in the multidivtime control file. The following prior distributions were used in these analyses: 100 mya (SD = 50 mya) for the expected time between tip and root if there were no constraints; 0.008 (SD = 0.004) substitutions per site per million year for the rate of the root node, based on the calculation by dividing the median distance between the ingroup root and the ingroup tips obtained from *Estbranches* by the time unit; 0.02 (SD = 0.02) for the parameter that determines the magnitude of autocorrelation per million years; and 100 mya for the largest value of the time unit between the root and the tips. We repeated each analysis twice to assure that Markov chains were long enough to converge.

The PL method is a semiparametric approach using rate smoothing to allow for robust estimation of node ages in the presence of rate variation between lineages (Sanderson, 2002). Ages of nodes in the tree were estimated using penalized likelihood rate smoothing under a truncated newton algorithm with the program r8s, version 1.60 (Sanderson, 2003; available at http://ginger.ucdavis.edu/r8s). A cross-validation analysis was performed to obtain the most likely smoothing parameter. Standard deviations (SD) associated with divergence times were calculated using nonparametric bootstrapping (Baldwin and Sanderson, 1998), repeating the dating procedure 100 times with 100 topologically identical trees with varying branch lengths obtained from 100 bootstrap matrices, the latter were generated using the program Seq-Gen, version 1.2.7 (Rambaut and Grassly, 1997). The divergence times were estimated on each tree as described and the resulting ages were used to calculate the variance in divergence time estimates.

Only a few fossils are reported for the Lamiales (Manchester, 1999). Fossils of *Fraxinus* L. (Oleaceae) are known from the Eocene Claiborne Formation of southeastern North America (Call and Dilcher, 1992) and have been recorded from the Oligocene (Meyer and Manchester, 1997) and the Miocene of the Pacific Northwest (Chaney and Axelrod, 1959). The oldest reliable *Fraxinus* fossil is from the late Eocene of North America (Magallón-Puebla et al., 1999; Manchester, 1999). Seeds of *Catalpa* have been reported from the early Oligocene of Oregon (Meyer and Manchester, 1997; Manchester, 1999). Because the fossil seeds are smaller than those of the extant *Catalpa*, its assignment to the genus may be questionable, but they certainly belong to Bignoniaceae (S. Manchester, University of Florida, Gainsville, personal communication). The oldest reliable Bignoniaceae fossil is a fruit with seeds from the late early Eocene of Washington State (Wehr and Hopkins, 1994; Pigg and Wehr, 2002; Wolfe et al., 2003). We constrained the Bignoniaceae node (node A in Fig. 2) with the minimum age as 49.4 mya (see Wehr and Hopkins, 1994; Wolfe et al., 2003). Alternatively, the *Catalpa-Macrocatalpa* node (node B in Fig. 2) was also constrained to a minimum age of 35 mya. The *Fraxinus-Osmanthus* node (node C in Fig. 2) was constrained to a minimum age of 37 mya. Because root age in the PL method is required, the Lamiales clade was assigned to be 74 or 97 mya based on the estimates by Wikström et al. (2001) and Bremer et al. (2004), respectively, to provide a range of estimation despite these estimates were arguable with limitations, such as the relatively poor calibrations.

We used dispersal–vicariance (DIVA) analysis (Ronquist, 1997) to infer the biogeographic diversification of *Phryma* and its close relatives on a tree estimated using combined ITS, ETS, and *trnL-F* data from taxa previously analyzed by Beardsley and Olmstead (2002), Beardsley et al. (2004), and Beardsley and Barker (2005). We were especially interested in inferring the ancestral area of the intercontinentally disjunct *Phryma* (Asia or North America or another area). Six areas of endemism were circumscribed according to the

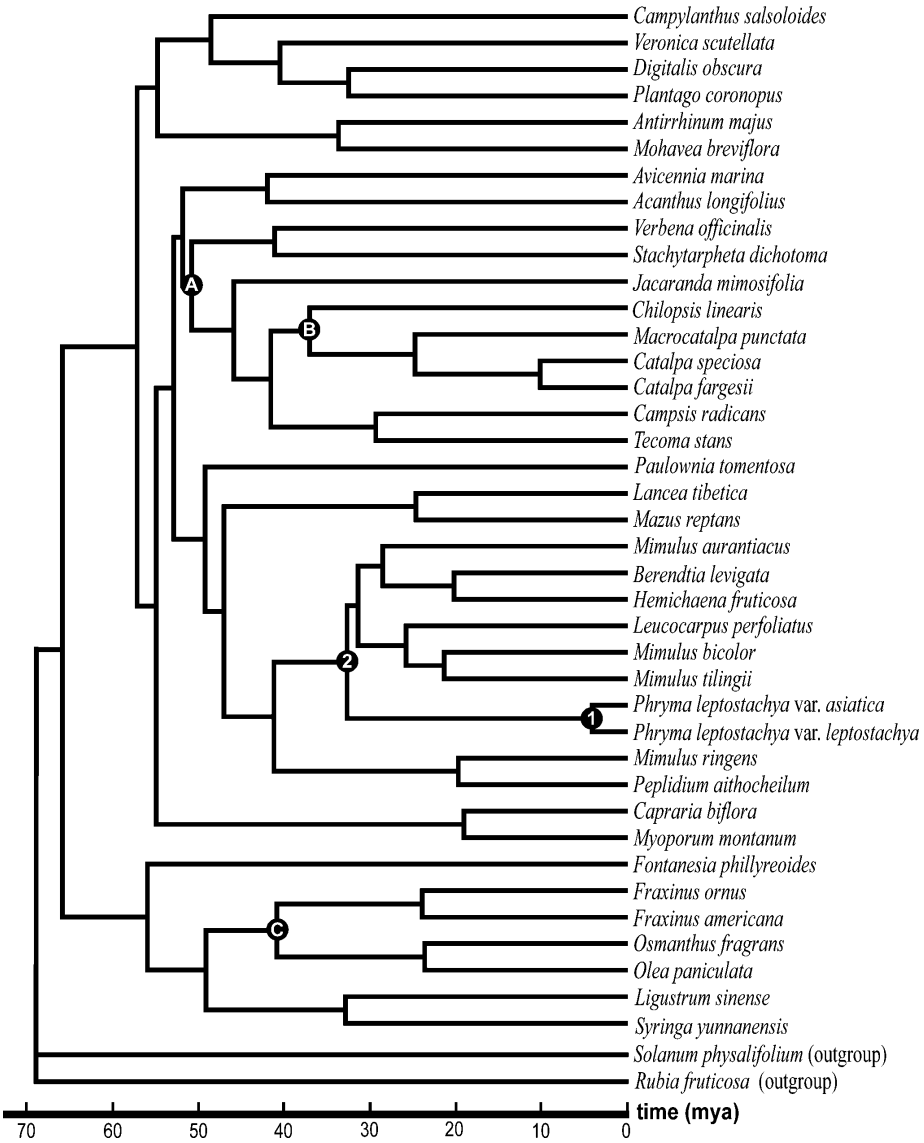

Fig. 2. Chronogram of *Phryma* and close relatives of the Lamiales based on the maximum likelihood tree of combined *rps16* and *trnL-F* data. Divergence times are shown using the Bayesian approach with internal minimum age constraints enforced (nodes A, B, and C are calibrating points and were constrained to 49.4, 35, and 37 mya, respectively based on fossils; nodes 1 and 2 are points of biogeographic interest with divergence times estimated in this study).

geographic distribution of Phrymaceae (Fig. 3): (A) Asia, (B) North America, (C) tropical America including South America, (D) Australia, (E) India, and (F) Africa. The DIVA analysis was implemented with the computer program DIVA, version 1.1 (Ronquist, 1996).

Morphological analysis—A total of 116 herbarium specimens from A, CAS, CDBI, F, KUN, PE, and US was measured in our analysis. This sampling scheme covered the entire distributional range of *Phryma* in both EA and ENA (Appendix 3). Only specimens with leaves, flowers, and fruits were measured in our study.

Phryma plants from EA and ENA are very similar morphologically (Hara, 1962). Plants from the two geographic regions may vary in the shape, size, and pubescence of leaves, and in some floral characters, such as the shape of upper lip of the corolla, and length of the upper spinulose calyx-lobes (Li, 2000). Characters used in our study were selected based on the variations enumerated by Hara (1962, 1966; Li, 2000) and our examinations of herbarium specimens. Twenty-three quantitative characters were measured, including nine leaf characters, 10 on flowers or inflorescences, three on fruits, and one on stems. Length and width were measured to the nearest millimeter from each specimen. One mature leaf approximately two to four nodes down from the inflorescences was measured from each specimen. Fully opened flowers near the top of the inflorescence and mature fruits near the bottom of the infructescence were measured on characters including the length of upper lip split of corolla, length of upper calyx lobes, and length and width of calyx tube and fruit. The pubescence on leaves and stems was documented (such as the number of hairs/0.25 cm^2 on lower leaf surface) under a Zeiss dissecting microscope (see Table 2).

One-way analysis of variance (ANOVA) was used to analyze the variance for each quantitative character with their distribution of EA and ENA as the grouping criterion using the computer program Minitab (version 12.23, Minitab, State College, Pennsylvania, USA). Dendrograms based on all specimens were produced by NTSYSpc, version 2.02h (Rohlf, 1998; Exeter Software, New York, New York, USA). Averages for each character were standardized to eliminate the effects of different scales of measurement using the default STAND procedure in NTSYSpc. Similarity matrices were prepared using the Jaccard's similarity coefficient. The cluster analysis was performed

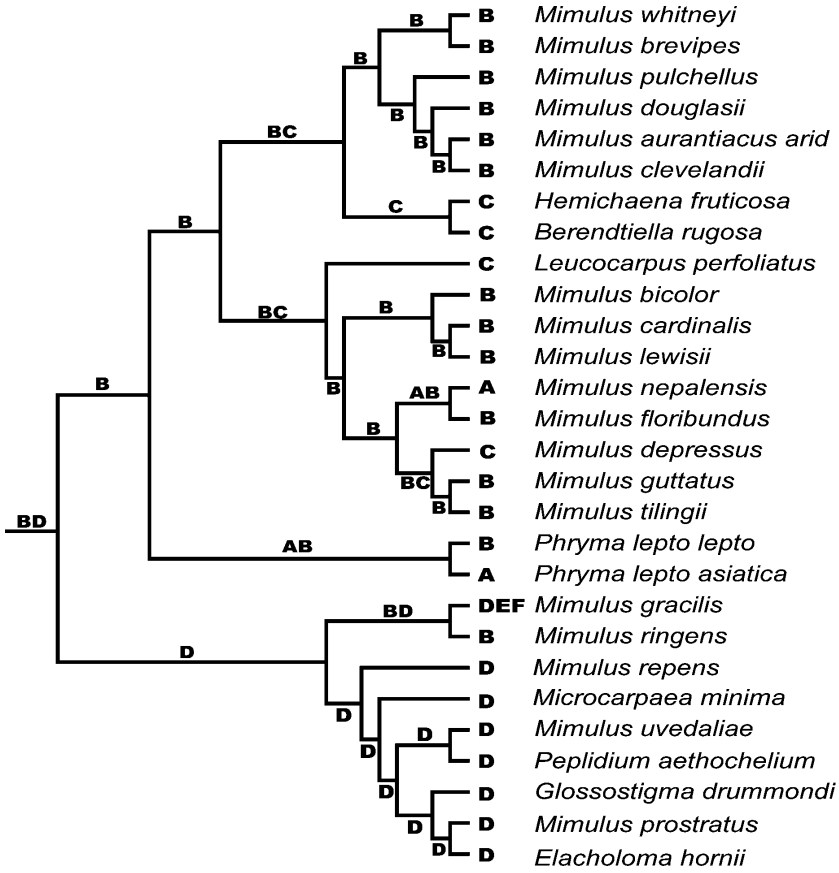

Fig. 3. Dispersal–vicariance analysis of Phrymoideae of Phrymaceae (A = Asia, B = North America, C = tropical America including South America, D = Australia, E = India, and F = Africa).

on the similarity matrix employing the unweighted pair-group arithmetic averages (UPGMA) algorithm (Sneath and Sokal, 1973).

Principal components analysis (PCA) was used to detect morphological variation between different populations and to analyze relationships between characters. PCA can help reveal unexpected relationships among a large number of variables into two or three new uncorrelated variables so that they retain most of the original information. The measurements for each character were also standardized to eliminate the effects of different scales of measurements for different characters. Similarity matrices using the Jaccard's similarity coefficient were then generated. Eigenvalue and eigenvector matrices were calculated from the similarity matrix. The standardized data were projected onto the eigenvectors of the correlation matrix and represented in a two-dimensional scatter plot. Plots of samples in relation to the first three principal components were constructed with populations designated as EA or ENA distribution. PCAs were also performed in NTSYSpc.

TABLE 1. Penalized likelihood (PL) and Bayesian dating estimates of divergence times (mya) of *Phryma* with standard deviations. PL1 and PL2 were estimated with the root of the Lamiales set as 74 and 97 mya, respectively.

Nodes in Fig. 2	Estimation method	Constraints on nodes A and C	Constraints on nodes A, B, and C
Node 1 (Asian and North American *Phryma*)	Bayesian	3.68 ± 2.25	3.93 ± 2.46
	PL1	3.84 ± 1.02	4.05 ± 1.07
	PL2	4.98 ± 1.32	5.23 ± 1.37
Node 2 (*Phryma* and its sister)	Bayesian	32.32 ± 4.46	32.91 ± 4.60
	PL1	36.20 ± 2.55	38.25 ± 2.50
	PL2	47.05 ± 3.36	49.35 ± 3.18

RESULTS

Molecular analysis—The ITS region ranged from 612 to 614 base pairs (bp) for all the populations of *Phryma*. The ITS-1 region had 16 variable nucleotide sites and one deletion within *Phryma*, and ITS-2 had eight variable sites and one deletion. The 5.8S rRNA gene was invariant among all populations sampled. Sequence alignment produced a data matrix of 627 aligned positions with *Mimulus aurantiacus* and *Lancea tibetica* as outgroups. A total of 841 bp of the *rps16* intron were obtained for all populations of *Phryma*. There was no length variation and only 11 nucleotide substitutions were detected. The *trnL-F* sequences ranged from 839 to 845 bp with two nucleotide substitutions and one deletion. Results of the partition homogeneity test for ITS vs. *rps16* and *trnL-F* showed that none of the data sets were significantly different from random pairwise partitions of the data. Therefore, we combined both the nrDNA and cpDNA data in subsequent analyses. In the combined ITS, *rps16*, and *trnL-F* data (2402 aligned positions) with *M. aurantiacus* and *L. tibetica* as outgroups, 338 sites were variable, 98 of which were parsimony-informative. Two most parsimonious trees (MPTs) were generated with a length of 396 steps, a consistency index (CI) of 0.97 (CI excluding uninformative characters = 0.90), a retention index (RI) of 0.96, and a rescaled consistency index (RC) of 0.92. The strict consensus and ML trees resulting from the combined data with bootstrap support and posterior probabilities is shown in Fig. 1. The phylogenetic analysis

TABLE 2. Descriptive statistics of morphological characters in eastern Asian and eastern North American samples of *Phryma* (mean ± SD).

Character	Eastern North America	Eastern Asia	*P*
Leaf length (cm)	11.28 ± 2.82	8.54 ± 2.31	*
Leaf width (cm)	5.90 ± 1.62	4.25 ± 1.22	*
Leaf length / width	1.94 ± 0.30	2.05 ± 0.44	0.103
Number of teeth on half leaf margin	13.47 ± 3.11	14.75 ± 2.88	0.024
Teeth number / leaf length	1.22 ± 0.26	1.81 ± 0.41	*
Inflorescence length (cm)	19.06 ± 3.63	18.56 ± 5.40	0.560
Flower number per inflorescence (No. pairs)	16.54 ± 3.73	16.45 ± 4.60	0.912
Width of inflorescence main axis at the base (mm)	0.98 ± 0.17	0.93 ± 0.18	0.11
Calyx tube length (mm)	2.65 ± 0.51	2.55 ± 0.42	0.246
Calyx tube width (mm)	1.13 ± 0.17	1.01 ± 0.16	*
Calyx tube length/width ratio	2.38 ± 0.48	2.56 ± 0.40	0.195
Bract length (mm)	1.79 ± 0.35	1.75 ± 0.35	0.533
Length of upper calyx lobes at anthesis (mm)	2.22 ± 0.34	1.81 ± 0.36	*
Length of upper calyx lobes at fruiting (mm)	2.58 ± 0.42	2.27 ± 0.35	*
Length of upper lip split of corolla (mm)	0.15 ± 0.11	0.38 ± 0.13	*
Fruit length (mm)	5.40 ± 0.87	4.93 ± 0.86	0.005
Fruit width (mm)	1.32 ± 0.26	1.14 ± 0.19	*
Fruit length/width	4.11 ± 0.80	4.35 ± 0.69	0.079
Stem pubescence (No. hairs/0.5 cm)	32.49 ± 37.07	43.75 ± 34.84	0.095
Pubescence of upper leaf surface (No. hairs/0.25 cm^2)	30.14 ± 17.30	44.19 ± 22.08	*
Pubescence of lower leaf surface (No. hairs/0.25 cm^2)	24.46 ± 16.83	40.09 ± 20.57	*
Pubescence on upper leaf midvein (No. hairs/0.5 cm)	45.98 ± 29.92	50.07 ± 22.68	0.410
Pubescence on lower leaf midvein (No. hairs/0.5 cm)	38.84 ± 29.14	52.81 ± 25.69	0.008

* Denotes significant difference at $P < 0.001$ level.

recovered two highly distinct clades within *Phryma* corresponding to ENA (BV = 100%) and EA (BV = 98%). In the EA clade, accessions from southwestern China (EA1, EA2, EA5, and EA10) and central and eastern China (EA6, EA7, EA8, and EA9) form two subclades. The populations from northeastern China (Jilin province, EA3), and Japan (EA11 and EA14) form a basal position (Fig. 1).

Pairwise distances among populations of *Phryma* were estimated and ranged 3.11–4.41% in the ITS sequences between EA and ENA populations. Intracontinental variation ranged 0–1.63% in the EA clade and 0–0.65% in the ENA clade. No sequence differentiation was detected in populations between EA1 and EA5 from Yunnan, China and between EA4 (Jilin of China) and EA13 (Honshu of Japan) in EA. Accessions of ENA2, ENA4, ENA6, ENA7, and ENA8 from ENA are identical in ITS sequence profiles. The combined *rps*16 and *trnL-F* data had a similar pattern of variation, but the variation was lower (0.54–0.71%) between the two continents.

Biogeographic analysis—Similar results from both Bayesian and PL divergence-time estimation using the concatenated cpDNA data are shown in Table 1 and Fig. 2. Bayesian dating estimates the minimum divergence time between the two intercontinental disjunct populations of *Phryma* as 3.68 ± 2.25 or 3.93 ± 2.46 mya (node 1 in Table 1 and Fig. 2), which yields time estimates in the late Tertiary (late Miocene to Pliocene). Using the smoothing value of 32 (root age = 74) or 100 (root age = 97) as obtained from the cross-validation procedure in the r8s program, the PL analysis suggested the minimum divergence time as 3.84 ± 1.02 to 5.23 ± 1.37 mya (Table 1). The split of *Phryma* and its sister group (sensu Beardsley and Olmstead, 2002) was estimated to have occurred at least 32.32 ± 4.46 or 32.91 ± 4.60 mya and 36.20 ± 2.55 to 49.35 ± 3.18 mya (node 2 in Table 1 and Fig. 2) with Bayesian and PL estimates, respectively. The DIVA analysis suggested the ancestral area of *Phryma* and its sister group to be North America (Fig. 3), when the possible ancestral area was constrained to be two areas (the minimum allowable option). Under this constraint, populations of *Phryma* from EA are inferred to have dispersed from North America to EA via the Bering land bridge (Fig. 4).

Morphometric analysis—Twenty-three characters were measured for 59 specimens from ENA and 57 from EA. Ten of the 23 quantitative characters are significantly different ($P < 0.001$) between the two intercontinental varieties (Table 2). For example, leaves from the ENA samples were both longer (ENA = 11.28 ± 2.82 cm, EA = 8.54 ± 2.31 cm) and wider (ENA = 5.90 ± 1.62 cm, EA = 4.25 ± 1.22 cm) (Table 2). The upper lobes of the calyx of the ENA plants are longer than those of EA at anthesis (ENA = 2.22 ± 0.34 mm, EA = 1.81 ± 0.36 mm) and at fruiting (ENA = 2.58 ± 0.42 mm, EA = 2.27 ± 0.35 mm). The upper lip of the corolla is subentire or emarginate in ENA plants, but it is always two-lobed in EA plants with a deeper split than the ENA plants (ENA = 0.15 ± 0.11 mm, EA = 0.38 ± 0.13 mm).

The first three principal components describe approximately 46.83% of the variation with eigenvalues of 4.66, 3.45, and 2.66. The first component accounts for 20.27% of the total variance, while the second component accounts for 15%. Characters contributing the most to the first component include leaf length and width, pubescence of upper and lower leaf surface, and teeth number on each side of the leaf. Characters contributing the most to the second component are calyx tube width, length of the upper lobe of calyx both at anthesis and at fruiting, and pubescence on the adaxial midvein of the upper leaf. The third component rests largely on inflorescence length, flower number per inflorescence, and number of teeth on the upper half of the leaf blade (on one side). Morphological differentiation between the Old and the New World accessions was found to be low in the multivariate analyses. Neither the PCA nor the UPGMA cluster analyses

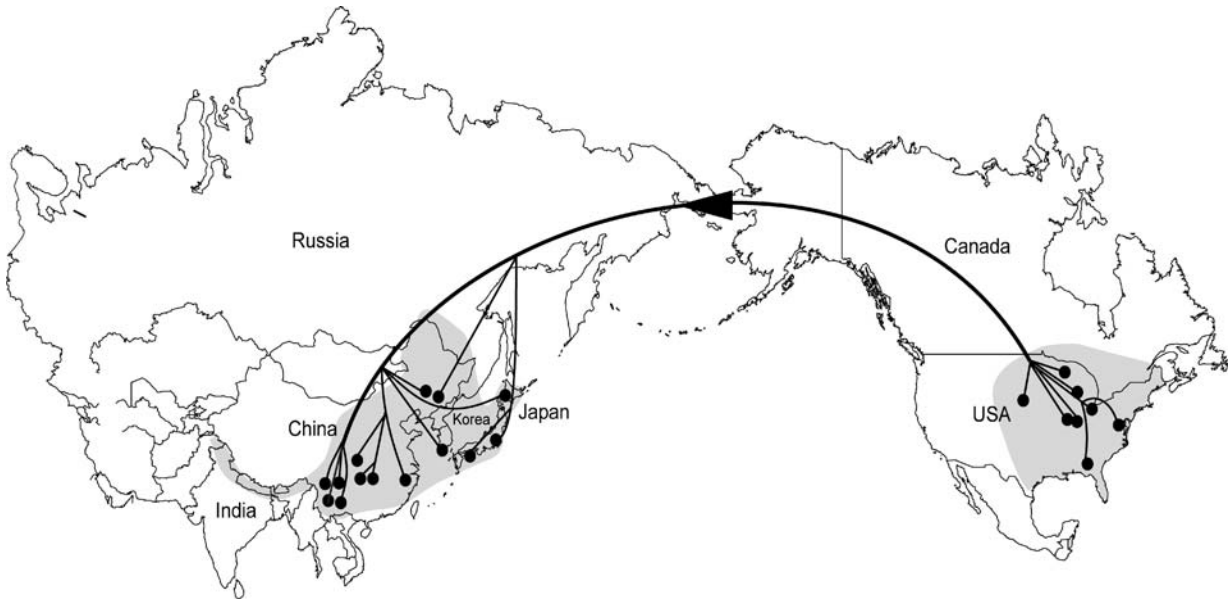

Fig. 4. Distribution map (shaded areas) of *Phryma* with sampling localities indicated as black dots. A schematic depiction of the phylogeographic migration route from North America to eastern Asia through the Bering land bridge and the diversification of the genus in both eastern Asia and eastern North America is included.

yielded distinct morphological groups corresponding to the two continents (Figs. 5 and 6). The PCA plots only showed a rough separation of the intercontinental populations with some overlapping.

DISCUSSION

Molecular divergence and phylogeography—The intercontinental populations of *Phryma* are generally recognized as a single species despite their wide geographic disjunction. Our molecular data indicate that the EA and ENA populations are well differentiated in terms of DNA sequence, though together they form an unambiguous clade. The combined sequences of ITS, *rps16*, and *trnL-F* data resolved two highly distinct monophyletic groups corresponding to the EA and ENA populations (Fig. 1). The pairwise sequence divergence between the two varieties was higher than that among populations within each continent for all ITS, *rps16*, and *trnL-F* markers. For the ITS regions, we obtained sequence divergence between the two varieties of 3.11–4.41%; a similar divergence (4.46%) was observed by Lee et al. (1996).

Based on our present sampling, the EA populations are more heterogeneous in ITS sequence variation than those in ENA (maximum divergence of 1.63% vs. 0.65%). The phylogeny based on DNA sequences revealed several geographic groupings within EA (Figs. 1 and 4). The populations from southwestern China (Yunnan and Sichuan provinces, EA1, EA2, EA5, and EA10) form a clade. Those from central and eastern China (Chongqing, Gansu, and Zhejiang provinces, EA6, EA7, EA8, and EA9) also form a group. Three accessions from Japan are separated into two groups, two of them (EA11 from Kyoto and EA14 from Shikoku) together with EA3 from Jilin of China form a basal position to the rest of the Asian specimens, and the other one is identical to those from Korea and Jilin of China (EA12 and EA4 in Fig. 1). The population from Illinois (ENA1) is sister to the rest of the ENA clade. Populations from Alabama, Indiana, and Virginia (ENA3, ENA5, and ENA7) grouped together with relatively low bootstrap support (BV = 55%, Fig. 1). However, no clear phylogeographic structure was detected among the ENA populations. The more pronounced sequence variation in EA *Phryma* may be due to higher amounts of geographic isolation resulting from the more heterogeneous topographies of EA (Wen, 1999) or to the extirpation of populations throughout much of the range in North America during recent glaciations. Our sampling in EA indeed included various terrains in three distinct regions: southwest China, Sino-Japanese Asia, and north-temperate Asia.

Ancient genus with relatively recent species disjunction—Our biogeographic analysis based on combined *rps16* and *trnL-F* sequence data using both the Bayesian dating and penalized likelihood methods suggests the divergence time of *Phryma* populations between EA and ENA to be at least 3.68 $\pm$ 2.25 to 5.23 $\pm$ 1.37 mya, in the late Tertiary (Table 1). Our analyses employed fossils from other taxa in the Lamiales as calibration points. Lee et al. (1996) reported the divergence time to be 12.35 mya (no estimate of error was provided by the authors) using ITS sequences with the nucleotide substitution rate of 3.9×10^{-9} per site per year or about 18.5–24.6 mya based on isozyme data. Xiang et al. (2000) suggested that the divergence time between the two *Phryma* varieties was 5.85 $\pm$ 2.66 mya based on *rbcL* sequences with a general molecular clock calibrated using fossils of *Cornus*. The ITS nucleotide substitution rate in Lee et al. (1996) was calibrated using the rate estimated from *Dendroseris*, a relatively distantly related taxon in the Asteraceae, which was based on the geological age of the Juan Fernandez islands (Sang et al., 1994). An indirect estimate without fossils, based on a distantly related group of oceanic-island plants, may have led to an underestimate of the nucleotide substitution rate by Lee et al. (1996).

Our analysis suggests an ancient origin of the stem-lineage

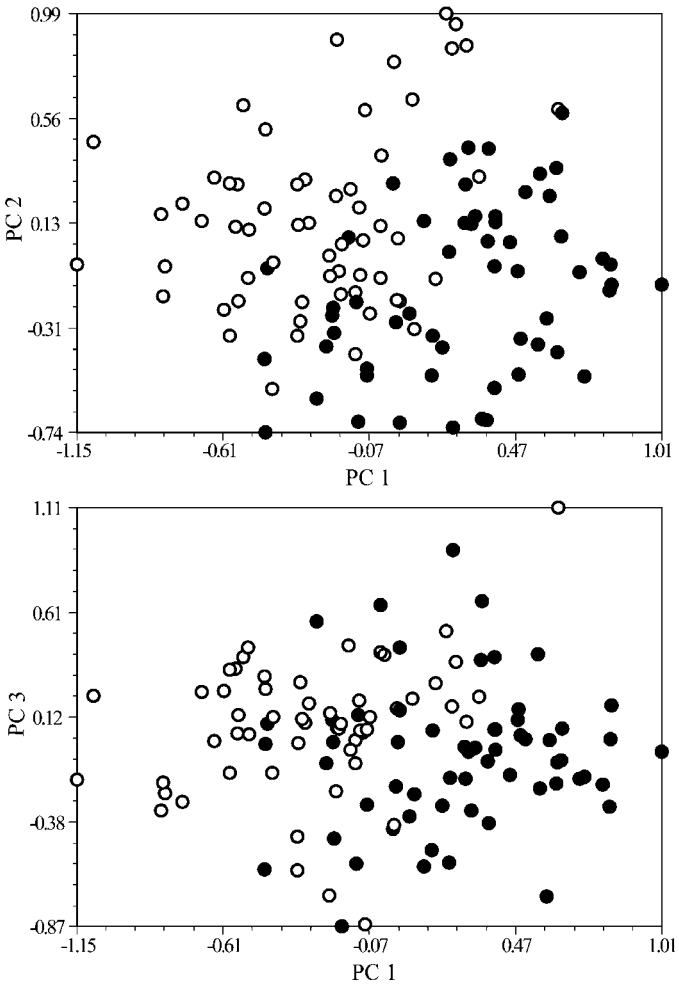

Fig. 5. Principal component analysis of morphological data from *Phryma*. Accessions are plotted according to the values of the first (*x*-axis) and the second (*y*-axis), and the first (*x*-axis) and the third (*y*-axis) components. (● = *P. leptostachya* var. *leptostachya* from eastern North America; ○ = *P. leptostachya* var. *asiatica* from eastern Asia).

leading to the genus *Phryma*, with the divergence time between *Phryma* and its sister group estimated as 32.32 ± 4.46 to 49.35 ± 3.18 mya (middle to the late Eocene, Table 1). The antiquity of *Phryma* was proposed by Hara (1966) based on its unique morphology. This finding is consistent with the hypothesis that the EA and ENA disjunct taxa are relicts of the temperate forests in the northern hemisphere that reached their maximum development during the Tertiary (Axelrod, 1975; Tiffney, 1985a; Manchester, 1999; Wen, 1999). On the other hand, the intercontinental disjunction of the two varieties is herein suggested to be much more recent, originating in the late Tertiary (late Miocene–Pliocene). A similar pattern was reported for the *Corylus* disjunction between EA and North America (Whitcher and Wen, 2001). Fossil data suggest an ancient origin of *Corylus* in the middle Eocene, yet the extant disjunct species had a relatively recent divergence in the late Tertiary (Whitcher and Wen, 2001).

Intercontinental migration—Our DIVA analyses supported the North American origin of *Phryma* and subsequent dispersal into EA (Fig. 3). In Phrymaceae, *Phryma* and at least five other genera are nested within *Mimulus* sensu lato (Beardsley and

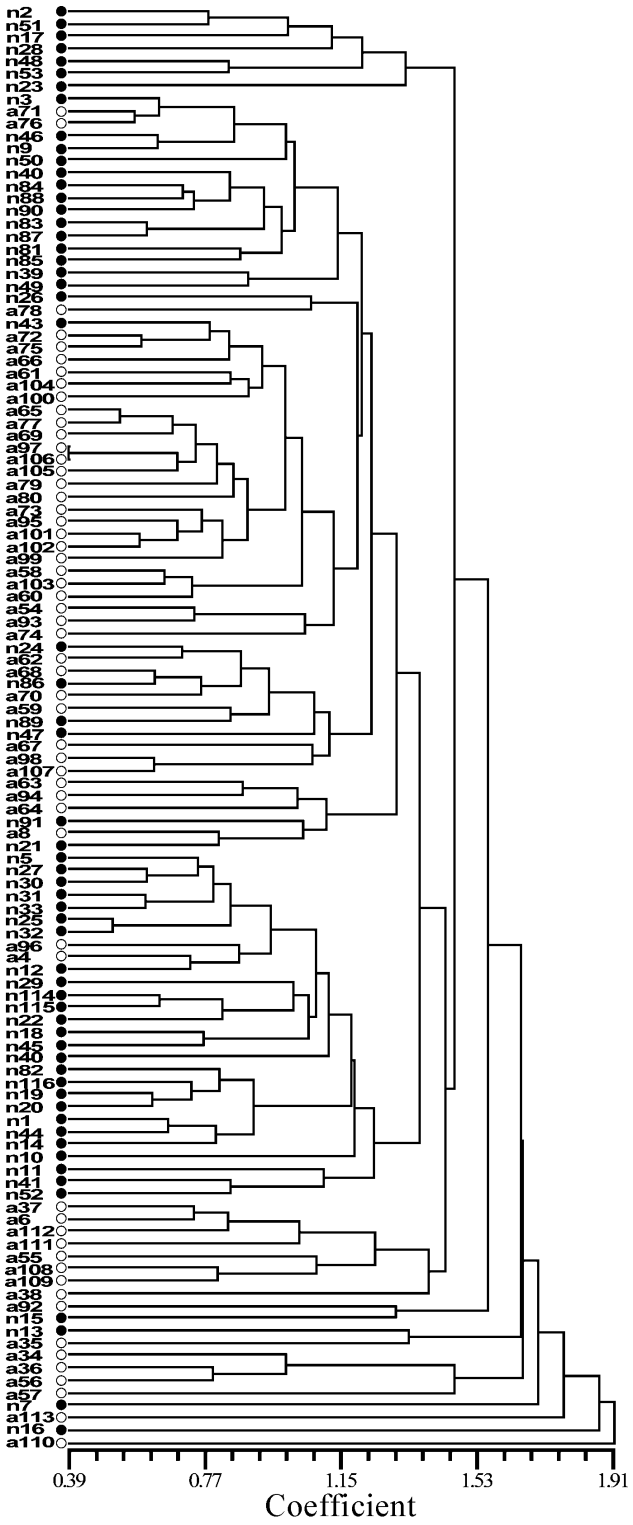

Fig. 6. UPGMA dendrogram of *Phryma* based on morphological data. Sample names beginning with "a" are from eastern Asia, and those with "n" are from eastern North America. (● = *P. leptostachya* var. *leptostachya* from eastern North America; ○ = *P. leptostachya* var. *asiatica* from eastern Asia).

Olmstead, 2002). *Mimulus* has a center of diversity in WNA, only five species are from EA (Hong, 1983; Beardsley and Olmstead, 2002). The genus shows a strong biogeographic connection between EA and North America with a clade of three Asian species derived from within a clade of WNA taxa that are found primarily in California and the Pacific Northwest (Beardsley and Olmstead, 2002). Also, the Asian *M. sessilifolius* has a close relationship to the WNA *M. dentatus* (Hong, 1983; Beardsley et al., 2004). The DIVA analysis suggests that North America is also more important for the early diversification of *Phryma* and its sister group than Asia.

Two populations from southern Japan (EA11 from Kyoto and EA14 from Shikoku) and one from northeastern China (EA3) form a basal grade relative to the rest of the EA clade, whereas populations from SW China and central and eastern China each form derived clusters of populations, suggesting that *Phryma* diversified in NE Asia first and migrated to the west.

The phylogeographic and DIVA results, in combination with the divergence time of the intercontinental populations, suggest that the current disjunct distribution of *Phryma* may be best explained by migration from North America into EA through the Bering land bridge. During the mid to late Tertiary, the Bering land bridge was suitable for exchanges of temperate deciduous plants and remained available for floristic exchanges until about 3.5 mya (Wen, 1999). Floristic migration via the North Atlantic land bridge was possible during the Paleocene and Eocene (Tiffney, 1985b) and was no longer viable by the middle Miocene (Parks and Wendel, 1990; Tiffney and Manchester, 2001).

Plants of *Phryma* usually are found in moist deciduous, or sometimes mixed deciduous and evergreen forests in EA and ENA (Ramana et al., 1983). Fossil evidence indicated that similar forests existed in WNA (Wolfe, 1975). Ancestors of *Phryma* may have been distributed in WNA during the development of "boreotropical flora," which reached the regions of high paleolatitudes in the northern hemisphere during the early Tertiary (Wolfe, 1972, 1975). Palynological evidence from Alaska and northwestern Canada also suggested that thermophilic taxa were abundant in the high latitudes during the period of around 15 mya, and temperate taxa became more important elements after 7 mya associated with the global climatic cooling in the late Tertiary (White et al., 1997; Graham, 1999). The disjunction in *Phryma* was estimated to be at least 3.68 ± 2.25 to 5.23 ± 1.37 mya. Thus, this estimate is consistent with a possible migration route through the Bering land bridge from North America to EA. The hooked persistent upper calyx lobes on *Phryma* fruits may have played an important role in facilitating the migration of *Phryma* to its present wide distribution and disjunction. Under this scenario, the ancestral diversity would have been greater in North America than in EA, but this is not reflected in current population differentiation. Extinction through much of its ancestral range due to continental glaciation, drying of the midcontinental region of North America, and mountain building in WNA, may explain the monophyly of populations in each geographic region obtained in our results.

Morphological differentiation and stasis—In our morphological analysis, 13 of the 23 characters were not significantly different ($P \geq 0.001$) between the EA and ENA samples (Table 2), including inflorescence length, number of flowers per inflorescence, number of teeth on the leaf margin, and stem pubescence. Ten quantitative characters were significantly different ($P < 0.001$) between the two intercontinental varieties. Five of the 10 characters are vegetative, including leaf length, width, number of teeth on the leaf margin per leaf length, and pubescence on leaf both upper and lower surface (Table 2). Specimens from ENA usually have larger leaves (length: 11.28 ± 2.82 vs. 8.54 ± 2.31 cm; width: 5.90 ± 1.62 vs. 4.25 ± 1.22 cm) than those from EA. Five floral characters were shown to be statistically different (Table 2). The upper sepals from EA *Phryma* are shorter than those from North America (1.82 ± 0.36 vs. 2.22 ± 0.34 mm at anthesis; 2.27 ± 0.35 vs. 2.58 ± 0.42 after anthensis). The upper lip of the corolla is subentire or emarginate in ENA plants (0.15 ± 0.11 mm), but is always two-lobed and more deeply split in EA ones (0.38 ± 0.13 mm). Thus, the characters that Hara (1962, 1966) mentioned as the major difference between plants from EA and ENA are also significantly different in our results, such as the leaf size, shape of upper lip of the corolla, and length of the upper spinulose calyx-lobes as discussed earlier. Nevertheless, most characters overlap significantly between the intercontinental populations. The great degree of morphological overlap led previous workers to emphasize the similarities among populations.

The principal component (Fig. 5) and cluster analyses (Fig. 6) based on morphological characters show that there is no clear geographic correlation with the morphological variation, in contrast to the molecular analyses (Fig. 1). Within these broader geographic regions, samples from the same geographic region were not more similar to each other than those separated by greater distances. Morphological variation thus appears to not be significantly structured in our analysis. Although the morphological similarity between the two varieties of *Phryma* has been highlighted for a long time (Li, 1952; Thieret, 1972; Li, 2000), the Old and New World populations were roughly separated into two groups in the PCA plots (Fig. 5). The UPGMA dendrogram (Fig. 6) also shows that many samples from each continent grouped together, but there was no clear separation between the two varieties. It is intriguing that the two taxa are so similar morphologically in spite of their high degree of molecular divergence.

The discordance of the molecular and morphological rates of evolution may be explained by morphological stasis. Morphological stasis has been proposed for a few EA and ENA disjunct taxa (see the introduction). Some disjunct taxa are polyphyletic or paraphyletic, suggesting that the morphological similarities in these groups may be attributable to convergence or symplesiomorphies (Wen, 1999). Among various possible explanations for stasis in morphology (Eldredge and Gould, 1972; Charlesworth et al., 1982; Williamson, 1987; Williams, 1992), a relatively constant environment with the concomitant action of stabilizing selection, might be the most plausible. Disjunct species of herbaceous plants with relictual distributions in both EA and North America appear to exhibit stasis in ecological traits (Ricklefs and Latham, 1992). The intercontinental populations of *Phryma* occupy similar habitats in rich mesic to moist, deciduous or mixed deciduous and evergreen forests in both ENA and EA (Thieret, 1972; Ramana et al., 1983; J. Wen, personal observation). The similar habitats may have contributed to maintaining their morphological similarity (i.e., convergence) for a long period of time (Parks and Wendel, 1990; Hoey and Parks, 1991; Wen, 1999; Bond et al., 2001).

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APPENDIX 1. Voucher information and GenBank accession numbers for taxa used in this study. A dash indicates the region was not sampled. Voucher specimens are deposited in the following herbaria: F = Field Museum of Natural History, KUN = Kunming Institute of Botany, Chinese of Academy of Sciences, US = United States National Herbarium, Smithsonian Institution, A = Arnold Arboretum, Harvard University, WTU = University of Washington.

***Taxon*. Abbreviation**: GenBank accessions: ITS, *rps*16, *trnL-trnF*; Source; *Voucher specimen*.

Phrymaceae

***Phryma leptostachya* var. *leptostachya* L. ENA1**: DQ533801, DQ532443, DQ532465; USA: Illinois; *Wen 7140* (F). **ENA2**: DQ533802, DQ532444, DQ532466; USA: Illinois; *Wen 7161* (F). **ENA3**: DQ533803, DQ532445, DQ532467; USA: Alabama; *Wen 7188* (F). **ENA4**: DQ533804, DQ532446, DQ532468; USA: Wisconsin; *Wen 7292* (F). **ENA5**: DQ533805, DQ532447, DQ532469; USA: Indiana; *Olmstead 90–003* (WTU). **ENA6**: DQ533806, DQ532448, DQ532470; USA: Nebraska; *Olmstead s.n.* (WTU). **ENA7**: DQ533807, DQ532449, DQ532471; USA: Virginia; *Wen 8548* (US). **ENA8**: DQ533808, DQ532450, DQ532472; USA: Minnesota; Moore 21814 (US). ***Phryma leptostachya* var. *asiatica* Hara EA1**: DQ533809, DQ532451, DQ532473; China: Yunnan, Yimeng; *Nie 102* (KUN, F). **EA2**: DQ533810, DQ532452, DQ532474; China: Sichuan, Yangyuan; *Yue 122* (KUN,F). **EA3**: DQ533811, DQ532453, DQ532475; China: Jilin; *Wen 5422* (F). **EA4** : DQ533812, DQ532454, DQ532476; China: Jilin; *Wen 5434* (F). **EA5**: DQ533813, DQ532455, DQ532477; China: Yunnan, Songming; *Wen 5757* (F). **EA6**: DQ533814, DQ532456, DQ532478; China: Gansu; *Wen 8035* (F). **EA7**: DQ533815, DQ532457, DQ532479; China: Chongqing; *Wen 8119* (F). **EA8**: DQ533816, DQ532458, DQ532480; China: Chongqing; *Wen 8156* (F). **EA9**: DQ533817, DQ532459, DQ532481; China: Zhejiang; *Nie and Meng 382* (KUN). **EA10**: DQ533818, DQ532460, DQ532482; China: Sichuan; *Nie and Meng 450* (KUN). **EA11**: DQ533819, DQ532461, DQ532483; Japan: Honshu, Kyoto; *Tsugaru and Murata 23846* (A). **EA12**: DQ533820, DQ532462, DQ532484; South Korea: Cheju-do; *Boufford et al. 25741* (A). **EA13**: DQ533821, DQ532463, DQ532485; Japan: Hokkaido; *Yonekura 95623* (A). **EA14**: DQ533822, DQ532464, DQ532486; Japan: Shikoku; *Takahashi 1815* (A).

Bignoniaceae

***Catalpa fargesii* Bur.** : —, DQ532491, DQ532488; China: Yunnan; *Wen 5705* (F). ***Chilopsis linearis* Sweet**: —, DQ532492, DQ532489; USA: Texas; *Wen 7278* (F). ***Macrocatalpa punctata* (Griseb.) Britton**: —, DQ532490, DQ532487; Cuba: Pinar del Rio; *Olmstead 96–131* (WTU).

APPENDIX 2. Samples of Lamiales examined in the study for estimating divergence times. A dash indicates the sequence was missing.

***Taxon*:** GenBank accessions: *rps16*, *trnL-trnF*; Reference.

***Acanthus longifolius* Host:** AJ431037, AJ430912; Bremer et al., 2002. ***Antirrhinum majus* L.**: AY492195, AJ492270; Albach et al., 2005, Mayer et al., 2003. ***Avicennia marina* (Forssk.) Vierh.:** AJ431038, AJ430913; Bremer et al., 2002. ***Berendtia levigata* Robinson & Greenm.:** AJ609208, AJ608615; Oxelman et al., 2005. ***Campsis radicans* Seem.:** —, AY695865; Chen et al., 2005. ***Campylanthus salsoloides* Webb:** AY492199, AY492173; Albach et al., 2005. ***Capraria biflora* L.:** AJ609198, AJ608608; Oxelman et al., 2005. ***Catalpa speciosa* (Warder) Engelm.:** AJ609197, AJ608599; Oxelman et al., 2005. ***Digitalis obscura* L.:** AY218799, AF486418; Albach and Chase, 2004, Albach et al., 2004. ***Fontanesia phillyreoides* Labill.:** AF225226, AF231818; Wallander and Albert, 2000. ***Fraxinus americana* L.:** AF225233, AF231825; Wallander and Albert, 2000. ***Fraxinus ornus* L.:** AF225240, AF231832; Wallander and Albert, 2000. ***Hemichaena fruticosa* Benth.:** AJ609179, AY575501; Oxelman et al., 2005, Beardsley et al., 2004. ***Jacaranda mimosifolia* D.Don:** AJ431039, AJ430914; Bremer et al., 2002. ***Lancea tibetica* Hook.f. & Thomson:** AJ609174, AF479003; Oxelman et al., 2005, Beardsley and Olmstead, 2002. ***Ligustrum sinense* Lour.:** AF225256, AF231847; Wallander and Albert, 2000. ***Leucocarpus perfoliatus* Benth.:** —, AF478998; Beardsley and Olmstead, 2002. ***Mazus reptans* N.E.Br.:** —, F479004; Beardsley and Olmstead, 2002. ***Mimulus aurantiacus* Curt.:** AJ609163, AF478982; Oxelman et al., 2005, Beardsley and Olmstead, 2002. ***Mimulus bicolor* Hartw. ex Benth.:** —, F478995; Beardsley and Olmstead, 2002. ***Mimulus ringens* L.:** —, AF479000; Beardsley and Olmstead, 2002. ***Mimulus tilingii* Regel:** —, AF478994; Beardsley and Olmstead, 2002. ***Mohavea breviflora* Coville:** AJ609223, AF479011; Oxelman et al., 2005, Beardsley and Olmstead, 2002. ***Myoporum montanum* R.Br.:** AJ431059, AJ430934; Bremer et al., 2002. ***Olea paniculata* Roxb.:** AF225276, AF231867; Wallander and Albert, 2000. ***Osmanthus fragrans* Lour.:** AF225278, AF231869; Wallander and Albert, 2000. ***Paulownia tomentosa* (Thunb.) Steud.:** AJ609153, AF479005; Oxelman et al., 2005, Beardsley and Olmstead, 2002. ***Peplidium aithocheilum* W.R.Barker:** —, AF479002; Beardsley and Olmstead, 2002. ***Plantago coronopus* L.:** AY218801, AY101937; Albach et al., 2004, Ronsted et al., 2002. ***Rubia fruticosa Ait.* (outgroup):** AF004078, AF102475; Andersson and Rova,1999, Struwe et al. 1998. ***Solanum physalifolium* Rusby (outgroup):** AY727449, AY727207; Shaw et al., 2005. ***Stachytarpheta dichotoma* (Ruiz & Pavon) Vahl:** AJ299259, AJ299260; Wallander and Albert, 2000. ***Syringa yunnanensis* Franch.:** AF225293, AF231883; Wallander and Albert, 2000. ***Tecoma stans* (L.) Juss. ex Kunth:** —, AY008826; Schwarzbach and McDade, 2002. ***Verbena officinalis* L.:** AF225295, AF231885; Wallander and Albert, 2000. ***Veronica scutellata* L.:** AY218823, AF486393; Albach and Chase, 2004, Albach et al., 2004.

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APPENDIX 3. Herbarium specimens examined for the morphometric analyses of *Phryma*. Specimens examined are from the following herbaria: A = Arnold Arboretum, Harvard University; CAS = California Academy of Sciences; CDBI = Chengdu Institute of Biology, Chinese Academy of Sciences; F = Field Museum of Natural History; KUN = Kunming Institute of Botany, Chinese of Academy of Sciences; PE = Institute of Botany, Chinese Academy of Sciences; and US = United States National Herbarium, Smithsonian Institution.

Taxon

Designation code of the specimen: Locality; *Voucher specimen* (herbarium).

***Phryma leptostachya* var. *asiatica* Hara**

1. a4: Japan, Bitchu, Izumi, Banzaimura; *G. Murata 27269* (US). **2. a6:** Japan, Shibokusa, Oshino; *F. Konta 11453* (A). **3. a8**: Nepal, Gandaki Zone, Gorkha; *M. Suzuki et al. 9470184* (A). **4. a34:** Korea, Chejn-Do; *I. C. Chung 3791* (F). **5. a35:** Korea, Kwangnung, Kyonggi-Do; *I. C. Chung 3119* (F). **6. a36**: Korea, Kumchon, Kyongsang-Pukto; *I. C. Chung 9470* (F). **7. a37:** Korea, Odae-San , Kangwon-Do; *I. C. Chung 3677* (F). **8. a38:** Japan, Yamanashi, Kobuchizawa; *M. Togashi 7389* (F). **9. a54:** China, Sichuan; *Vegetation Group 13993* (CDBI). **10. a55:** China, Heilongjiang; *P. Z.Guo 75289* (CDBI). **11. a56:** China, Dalian; *Wang and Liu 955* (PE). **12. a57:** China, Jilin, Mt. Changbai; *Lin 449* (PE). **13. a58:** China, Beijing; *Wang et al. 293* (PE). **14. a59:** China, Hebei; *X. Y. Liu 802* (PE). **15. a60:** China, Jiangsu; *S. H. Mao 205* (PE). **16. a61:** China, Zhejiang; *Q. Long8786* (PE). **17. a62**: China, Zhejiang; *South Medicine Group 109* (PE). **18. a63:** China, Jiangxi; *Anonymous 94194* (PE). **19. a64:** China, Hunan; *L. H. Liu15836* (PE). **20. a65:** China Guizhou, Xingyi; *X. An 498* (PE). **21. a66:** Russia; *Coop s.n.* (PE). **22. a67:** China, Yunnan Yongde; *Liu and Yang 5444* (KUN). **23. a68:** China, Yunnan, Qiaojia; *B. X. Sun 886* (KUN). **24. a69:** China, Yunnan, Kunming; *Feng 10839* (KUN). **25. a70:** China, Hebei; *X. Y. Liu 802* (KUN). **26. a71:** China, Jilin, Yichuan; *C. N. Liu 7821* (KUN). **27. a72:** China, Liaoning, Chenyang; *W. Wang 225* (KUN). **28. a73:** China, Jiangsu, Piaoyang; *F. D. Liu 2630* (KUN). **29. a74:** China, Hunan, Sangchi; *Xiang Qiong Group 3625* (KUN). **30. a75:** China, Jiangxi, Guixi; *M. X. Nie and S. S. Lai 3968* (KUN). **31. a76:** China, Yunnan, Biyang; *Zhong Dian Group 4071* (KUN). **32. a77:** China, Yunnan, Bijiang; *S. G. Wu 8572* (KUN). **33. a78:** China, Yunnan, Eryuan; *B. Y. Qiu 61045* (KUN). **34. a79:** China, Guizhou, Yongjiang; *Z. P. Jian et al. 51695* (KUN). **35. a80:** China, Guizhou, Jiangkou; *Xiang Qiong Group 2501* (KUN). **36. a92:** China, Yunnan, Songming; *Sino-American Botanical Expedition to Yunnan China 1407* (KUN). **37. a93:** China, Shanxi; *P. Y. Li 8479* (KUN). **38. a94:** China, Yunnan, Qiubei; *H. Li 312* (KUN). **39. a95:** China, Guizhou, Shiqian Xian; *Mt. Wulinshan Expedition 2192* (KUN). **40. a96:** China, Guizhou, Shuojian; *Qian Nan Group 3062* (KUN). **41. a97:** China, Guizhou; *S. W. Ding 90623* (KUN). **42. a98:** China, Yunnan, Songming; *X. Zhou 1309* (KUN). **43. a99:** China, Yunnan, Heqing; *R. C. Qiong 24213* (KUN). **44. a100:** China, Shandong, Qingdao; *Z. Y. Wu and C. C. Lu 8614* (KUN). **45. a101:** China, Yunnan, Biyang; *Zhong Dian Group 63–4071* (KUN). **46. a102:** China, Jiangxi; *Anonymous 4171* (KUN). **47. a103:** China, Jiangxi, Mt. Lushan; *M. X. Nie 7537* (KUN). **48. a104:** China, Hunan, Yongxun; *L. H. Liu 9527* (KUN). **49. a105:** China, Shanxi, Fuping Xian; *K. J. Fu 878* (KUN). **50. a106:** China, Sichuan, Honghua; *Chuanjing Group 1395* (KUN). **51. a107:** China, Xizang, Jilong; *Qingzang Group 6909* (KUN). **52. a108:** Japan, N. Honshu, Miyagi; *Y. Tateishi et al. 14204* (A). **53. a109:** Japan, Hyogo, Yabu-gun, Oya-cho; *D. E. Boufford et al. 19573* (A). **54. a110:** South Korea, Nam-Cheju-gun; *D. E. Boufford et al. 25741* (A). **55. a111:** Japan, Kyoto, Ishizukuri-cho; *S. Tsugaru & Murata 23846* (A). **56. a112:** Japan, Kitami-Fuji Hokkaido Nippon; *K. Uno 2596* (A). **57. a113:** Japan, Miyagi, Sendai-hai; *T. Kurosawa 5081* (A).

***P. leptostachya* var. *leptostachya* L.**

58. n1: Canada, Quebec, La Trappe; *P. Louis-Marie s.n.* (US). **59. n2**: USA, Illinois, Kane Co.; *F. A. Swink 1615* (F). **60. n3:** USA, North Carolina, Rowan Co.; *A. A. Heller s.n.* (F). **61. n5:** USA, Pennsylvania, Chestnut Hill; *J. K. Small s.n.* (F). **62. n7:** USA, New York; *E. Hunt s. n.* (F). **63. n9:** USA, North Carolina, New Bern; *R. K. Godfrey & W. B. Fox 49544* (US). **64. n10:** USA, Kansas, Neodesha; *W. H. Horr 231* (US). **65. n11:** USA, Texas, Red River Co.; *C. L. Lundell 14000* (US). **66. n12:** USA, Tennessee, Lincoln Co.; *Anonymous s.n.* (US). **67. n13:** USA, Illinois, Elash City Park; *L. Bohs 1949* (F). **68. n14:** Canada, Quebec, vicinity of Ottawa; *B. Rolland 6186* (US). **69. n15:** USA, North Dakota; *L. Leeds s.n.* (US). **70. n16:** USA, Illinois, Chicago; *F. Gates 592* (F). **71. n17:** USA, Illinois, Chicago; *H. F. Jaeger s.n.* (F). **72. n18:** USA, Florida, Leon Co.; *R. M. Harper 3750* (US). **73. n19:** USA, Nebraska, Peru; *J. H. Winter 47* (US). **74. n20:** USA, South Dakota, Roberts Co.; *W. H. Over 14408* (US). **75. n21:** USA, Texas; *B. C. Tharp 2655* (US). **76. n22:** USA, Minnesota, Wilkin Co.; *J. M. Moore 21814* (US). **77. n23:** USA, Illinois, Grundy Co.; *T. G. Lammers 8777* (F). **78. n24**: USA, Pennsylvania, Newville; *N. V.*

Haynie s.n. (F). **79. n25:** USA, Michigan, Washtenaw Co.; *F. J. Hermann 9079* (F). **80. n26:** USA, Illinois, Cook Co.; *R. W. Du Ball 80* (F). **81. n27:** USA, Illinois, Cook Co.; *S. F. Glassman 5368* (F). **82. n28:** USA, Ohio, Lakeside Co.; *C. S. Mead 3777* (F). **83. n29:** USA, Kentucky, Edmonson Co.; *A. King 109* (F). **84. n30:** USA, Ohio, Vinton Co.; *D. E. O'Dell 1001* (F). **85. n31:** USA, Illinois. Du Page Co.; *F. A. Swink 267* (F). **86. n32:** USA, Missouri, Taney Co.; *J. A. Steyermark 40055* (F). **87. n33:** USA, Oklahoma, Platt National Park; *G. M. Merrill & W. M. A. Hagan 697* (F). **88. n39:** USA, Minnesota, Steele Co.; *T. G. Lammers 9494* (F). **89. n40:** USA, Virginia, near Luray; *E. S. Steele & Mrs. Steele 204* (US). **90. n41:** USA, Virginia, Shenandoah National Park; *E. H. Walker 2915* (US). **91. n42:** USA, Missouri, Laclede Co.; *D. Moore s.n.* (F). **92. n43:** USA, Texas; *J. Reverchon s.n.* (F). **93. n44:** USA, Wisconsin, vicinity of Delavan; *N. Hollister 101* (US). **94. n45:** USA, Arkansas; *D. Demaree 6973* (US). **95. n46:** USA, Indiana; *J. N. Nose s.n.* (F). **96. n47:** USA, Kansas, Barber Co.; *J. Barrell 65–74* (US). **97. n48:** USA, Illinois, Athens, Menard Co.; *E. Hall s.n.* (F). **98. n49:** USA, Lake Michigan; *O. E. Lansing 664* (F).**99. n50:** USA, North Carolina, Polk Co.; *D. C. Peattie 1016* (F). **100. n51:** USA, Illinois; *A. W. De Selm 365* (F). **101. n52:** USA, Kansas, Cherokee Co.; *R. L. McGregor 15793* (US). **102. n53:** USA, Illinois, Peoria Co.; *J. T. Stewart s.n.* (F). **103. n81:** USA, Illinois, De Kalb Co.; *E. K. Abbott s.n.* (CAS). **104. n82:** USA, Nebraska, Loup Co.; *S. Stephens 6861* (CAS). **105. n83:** USA, North Carolina, McDowell Co.; *S. W. Leonard et al. 1795* (CAS). **106. n84:** USA, Kentucky, Carter Co.; *F. A. Gilbert 873* (CAS). **107. n85:** USA, Louisiana; *E. J. Palmer 7991* (CAS). **108. n86:** USA, Ohio, Butler Co.; *T. Cobbe 118* (CAS). **109. n87:** USA, Ohio, Cincinnati; *C. G. Lloyd 18727* (CAS). **110. n88:** USA, South Dakota, Deuel Co.; *R. R. Halse s.n.* (CAS). **111. n89:** USA, Iowa, Fayette; *B. Frink 227* (CAS). **112. n90:** USA, Oklahoma; J.*Clemens 11783* (CAS). **113. n91:** USA, North Carolina; *X. W. Li 254* (KUN). **114. n114**: USA, Ohio, Champaign, Shaffers woods; *E. C. Leonard 1690* (US). **115. n115**: USA, Iowa, Bimira; *M. P. Somes 3460* (US). **116. n116**: USA, Michigan, Washtenaw; *F. J. Hermann 9079* (US).