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MOLECULAR PHYLOGENETIC SYSTEMATICS AND BIOGEOGRAPHY OF TRIBE NEILLIEAE (ROSACEAE) USING DNA SEQUENCES OF CPDNA, RDNA, AND *LEAFY*[1]

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A phylogeny of the tribe Neillieae (Rosaceae), which comprises *Neillia*, *Stephanandra*, and *Physocarpus*, was reconstructed based on nucleotide sequences of several regions of cpDNA, the ITS and ETS regions of rDNA, and the second intron of *LEAFY*, to elucidate relationships among genera and species in Neillieae and to assess the historical biogeography of the tribe. Phylogenetic analyses indicated that *Physocarpus* and *Neillia-Stephanandra* were strongly supported as monophyletic and suggested that *Stephanandra* may have originated by hybridization between two lineages of *Neillia*. Dispersal-vicariance analyses suggested that the most recent common ancestor of Neillieae may have occupied eastern Asia and western North America and that *Physocarpus* and *Neillia-Stephanandra* may have been split by an intercontinental vicariance event in the early Miocene. The biogeographic analyses also suggested that species of *Neillia* and *Stephanandra* diversified in eastern Asia, whereas in *Physocarpus* one dispersal event from western North America to eastern Asia occurred. Two divergent types of *LEAFY* sequences were found in the eastern North American species, *P. opulifolius*, but only one type was present in each plant. The two types of sequences may represent homeologous genes that originated by hybridization between *P. capitatus* and *P. monogynus*, both western North American species.

Key words: biogeography; chloroplast DNA; hybridization; Neillieae; phylogeny; ribosomal DNA; second intron of *LEAFY*.

The plants in the Northern Hemisphere exhibit several interesting biogeographic patterns of intercontinental disjunction (Raven, 1972; Wood, 1972). The eastern Asian–eastern North American disjunct distribution pattern is perhaps the most remarkable one among these and has been the subject of numerous taxonomic, biogeographic, and evolutionary studies for more than a century (Boufford and Spongberg, 1983; Wen, 1999). The distribution pattern has been explained as part of a larger pattern in the Northern Hemisphere, which resulted from the range restriction of once widespread ancestral populations across the Northern Hemisphere in response to geologic and climatic changes (Tiffney, 1985a, b; Wen and Stuessy, 1993; Qiu et al., 1995; Graham, 1999; Wen, 1999; Xiang et al., 2000). During the past decade, a number of plant groups has been investigated using a phylogenetic framework (e.g., Sang et al., 1997; Wen et al., 1998; Li et al., 2000; Manos and Stanford, 2001; Allen et al., 2003), and these studies have provided insight into the dynamic biogeographic history of the Northern Hemisphere. A recent biogeographic investigation by Xiang et al. (1998) attempted to identify a general model of relationships for several disjunct genera with distributions in eastern Asia and both eastern and western North America. While they have demonstrated a congruent pattern of relationship in which North American species form a clade that is sister to eastern Asian species, this common pattern in different plant groups, without information on their respective divergence times, does not necessarily indicate that they shared the same biogeographic history. More recently, a group of phylogenetic systematists, paleobotanists, and biogeographers attempted to assemble a variety of lines of evidence—phylogenetic, paleontological, and current distribution—to develop a complete understanding of the historical biogeography of the Northern Hemisphere (Manos and Donoghue, 2001). Along this line of research, Manos and Donoghue (2001) and Donoghue et al. (2001) have emphasized that a modern synthesis of historic biogeography of the Northern Hemisphere requires more detailed study of individual clades in a robust phylogenetic framework and more direct estimation of divergence times with accurate age estimation methods.

The tribe Neillieae (Rosaceae), comprising three taxonomically difficult genera, *Neillia* D. Don, *Physocarpus* (Cambess.) Raf., and *Stephanandra* Siebold & Zucc. (Maximowicz, 1879; Schulze-Menz, 1964), is an appropriate system for studying the historical biogeography of the Northern Hemisphere. Neillieae is distributed in eastern Asia and both western and eastern North America. While *Neillia*, *Stephanandra*, and *P. amurensis* are distributed in eastern Asia, *P. alternans*, *P. capitatus*, *P. malvaceus*, and *P. monogynus* are found in western North America and *P. opulifolius* occurs in eastern North America. Monophyly of Neillieae has been strongly supported by chloroplast DNA (cpDNA) sequence data, including *rbcL* (Morgan et al., 1994) and *matK* and *trnL-trnF* genes (Potter et al., 2002). Morphologically, members of Neillieae are characterized by lobed leaves with persistent or de-

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TABLE 1. Morphological characteristics of the three genera in Neillieae.

Character	*Neillia*	*Physocarpus*	*Stephanandra*
Inflorescence	Raceme, panicle	Corymb	Panicle
Hypanthium	Campanulate, cylindrical	Cupulate	Cupulate
Carpel dehiscence	Along ventral suture	Along ventral and dorsal sutures	Along ventral suture
No. of carpels	1 (rarely 2–5)	1–5	1
No. of seeds	2–10	2–5	1–2
Stellate trichomes	Absent except for *N. uekii*	Present	Absent
Glandular hairs on hypanthium in fruits	Present	Absent	Absent

ciduous stipules and ovoid shiny seeds with copious endosperm (Vidal, 1963). The total number of species of this tribe is relatively small (18 species), making it amenable to analysis using different kinds of character systems and phylogenetic methods. To date, the historical biogeography of Neillieae has not been studied.

In addition, Neillieae has been in need of a comprehensive systematic study using modern methods to analyze both molecular and morphological data. The morphological characters used to distinguish each genus often vary within as well as among genera (Table 1), and because of different interpretations of the morphological variation by many taxonomists, conflicting classification schemes have been proposed. For instance, Bentham and Hooker (1865), Greene (1889), and Jones (1893) treated *Physocarpus* as part of *Neillia*, with *Stephanandra* as a separate genus, whereas Kuntze (1891) classified all species of Neillieae in *Physocarpus*. Although many modern authors recognize three genera in Neillieae (Rehder, 1940; Schulze-Menz, 1964; Robertson, 1974; Takhtajan, 1997), no comprehensive systematic or phylogenetic study of all species has been made until now. Vidal (1963) and Cullen (1971) published revisionary studies of *Neillia* and briefly discussed morphological relationships among the three genera. Their studies, however, concentrated on *Neillia* only, and the characteristics were not evaluated phylogenetically. Both *Physocarpus* and *Stephanandra* have been treated in regional floristic manuals (Rydberg, 1908; Ohwi, 1965; Fernald, 1970; Yu and Ku, 1974; Gleason and Cronquist, 1991; Holmgren, 1997).

Oh and Potter (2003) examined the phylogenetic utility of the second intron of *LEAFY* in *Neillia* and *Stephanandra*, and compared the intron sequence data with chloroplast *trnL-trnF, trnD-trnT, matK-trnK,* and the nuclear internal transcribed spacer (ITS) sequence data. They demonstrated that the *LEAFY* intron is the most variable and phylogenetically useful of these regions for reconstructing the phylogeny of *Neillia* and *Stephanandra*. For this paper, we expanded gene sampling in those two genera by adding two regions from the chloroplast genome and the nuclear external transcribed spacer (ETS) region, and we determined all of the sequences from all species of *Physocarpus* in order to elucidate the phylogenetic relationships across Neillieae and to investigate the historical biogeography of the tribe based on the phylogenetic framework.

The sequences used in this study are divided into three groups, here designated molecular character systems. The first of these comprises sequence data of five regions of cpDNA. This includes sequences of the *trnL-trnF, trnD-trnT, psbA-trnK,* and *matK-trnK* regions, which have been widely used as valuable source of data for studying phylogenetic relationships at the specific and generic levels in angiosperms (Mort et al., 2002; Smedmark and Eriksson, 2002; Miller et al., 2003).

The second character system comprises sequence data for the ETS region in addition to ITS of nuclear ribosomal DNA (rDNA). While the ITS region has been widely used for elucidating phylogenetic relationship among closely related species in angiosperms (reviewed by Baldwin et al., 1995), the ETS region, flanked by the nontranscribed spacer (NTS) and 18S ribosomal gene, has not been used as widely as ITS because general primers are not available in most groups of angiosperms. ETS has, however, been used as a valuable source of data for phylogenetic studies at lower taxonomic levels in Asteraceae (Baldwin and Markos, 1998; Linder et al., 2000; Markos and Baldwin, 2001; Lee et al., 2003; Morgan, 2003; Saar et al., 2003), Cyperaceae (Starr et al., 2003), Fabaceae (Bena et al., 1998), and Malvaceae (Andreasen and Baldwin, 2001). Phylogenetic studies using ETS sequences have shown that the ETS region has a higher percentage of phylogenetically informative characters than ITS and, when combined with ITS data, ETS data improved phylogenetic resolution and increased bootstrap support compared to a phylogeny based on the ITS region alone (Baldwin and Markos, 1998; Markos and Baldwin, 2001; Morgan, 2003).

The third molecular character system is derived from *LEAFY*, a nuclear homeotic gene that regulates the establishment of floral meristem identity and flowering time in *Arabidopsis* (Weigel, 1995; Blázquez et al., 1997). The gene is distributed in all plants including mosses, ferns and "fern allies," gymnosperms, and angiosperms (Frohlich and Parker, 2000; Himi et al., 2001). Phylogenetic analyses of amino acid sequences of *LEAFY* suggest that the gene was duplicated on the stem lineage leading to seed plants, but that one copy was lost in angiosperms, making it a single-copy gene in diploid angiosperms (Frohlich and Parker, 2000; Himi et al., 2001). The nucleotide sequences of the second intron of the gene have been used in phylogenetic analysis of *Amorphophallus* (Grob et al., 2004), *Fagopyrum* (Nishimoto et al., 2003), *Gnetum* (Won and Renner, 2003), *Isoëtes* (Hoot and Taylor, 2001), and *Sphagnum* (Shaw et al., 2003), as well as in our previous analyses of *Neillia* and *Stephanandra* (Oh and Potter, 2003).

MATERIALS AND METHODS

Taxon sampling—All six species of *Physocarpus* (Poyarkova, 1939; Robertson, 1974; Rosatti, 1993; Holmgren, 1997), all three of *Stephanandra* (Ohwi, 1965; Yu and Ku, 1974), and seven of nine species of *Neillia* (Cullen, 1971; Yu and Ku, 1974) were included in this study. The two species of *Neillia* (*N. rubiflora* D. Don and *N. serratisepala* H. L. Li) not included in this study are rare species for which only a small number of collection records are known (Cullen, 1971). In most cases, at least two populations per species were analyzed (Appendix 1, see Supplemental Data accompanying online version of this article). Plant materials were identified based on original species

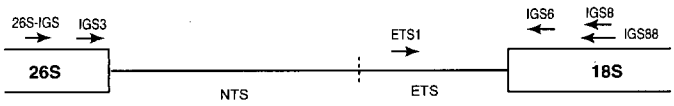

Fig. 1. Schematic diagram of ETS region, showing approximate location and orientation of primers. Ribosomal DNA coding regions are shown as boxes and the intergenic spacer, which includes NTS and ETS, is shown as the line connecting the coding regions. Because we did not determine the boundary sequence between NTS and ETS, the boundary is shown to represent the general structure of the regions. Drawings are not to scale.

descriptions and examination of the type specimens. Herbarium specimens from A, BM, CS, DAV, E, GH, JEPS, K, KPM, L, MO, NEBC, NY, P, PE, POM, UC, and UMO (Holmgren et al., 1990) were also used to examine morphological variation.

Phylogenetic analyses of Rosaceae based on various nucleotide sequence data have not resolved the sister group of Neillieae (Morgan et al., 1994; Potter et al., 2002). We used *Lyonothamnus* and *Vauquelinia* as outgroups because sequences of rDNA and cpDNA from these two taxa are easily aligned to those from Neillieae. *Lyonothamnus* is sister to the large clade in which Neillieae is nested, and *Vauquelinia* is nested in the sister clade of the tribe Neillieae (Morgan et al., 1994; Potter et al., 2002).

Gene sampling—We examined three regions of DNA: (1) several regions of chloroplast DNA (*trnL-trnF, trnD-trnT, matK-trnK,* and *psbA-trnK*), (2) spacer regions of nrDNA (ITS and ETS), and (3) the second intron of *LEAFY*.

Each region was amplified via polymerase chain reaction (PCR) from total DNA isolated from fresh or silica gel-dried young leaves using a DNeasy Plant Mini kit (Qiagen, Valencia, California, USA). For two accessions (*N. sparsiflora* and *P. alternans* 175), we extracted total DNA from herbarium specimens using the CTAB method (Doyle and Doyle, 1987).

Primer sequences, PCR conditions, cloning, and sequencing procedures for most of the regions (*trnL-trnF, trnD-trnT, matK-trnK,* ITS, and the second intron of *LEAFY*) are described in Oh and Potter (2003), while those for the ETS region are described separately later. The chloroplast *psbA-trnK* region was amplified using trn2 and psbA3 primers (Appendix 2, see Supplemental Data accompanying online version of this article) and was sequenced in both directions with the same PCR primer set. Nucleotide sequences of all regions were directly sequenced from PCR products except for *LEAFY*, in which sequences were primarily determined via cloning (Oh and Potter, 2003). Nucleotide sequences of *trnL-trnF, trnD-trnT, matK-trnK,* ITS, and the second intron of *LEAFY* from *Neillia* except for *N. sparsiflora, Stephanandra, P. amurensis,* and *P. capitatus* accession 082 were derived from Oh and Potter (2003); all other sequences were determined in this study (GenBank accession numbers are in Appendix 1).

All sequences were determined at the Division of Biological Sciences sequencing facility on the UC Davis campus, which uses an ABI PRISM 377 DNA Sequencer or an ABI PRISM 3100 Genetic Analyzer (PE Biosystems, Foster City, California, USA). Sequences were edited in Sequencher version 4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA), and IUPAC ambiguity symbols were used for uncertain and polymorphic sites.

Two divergent types of cloned sequence of the second intron of *LEAFY* were found in *P. opulifolius*, but only one of the two types, not both, was discovered in each accession (see Results). The two types differed in the number of *Xba*I cleavage sites. To test the possibility that both types were present in PCR products but that only one was selected in the cloning procedure, 1 μg of PCR products was digested with *Xba*I for each accession, and the digested DNA was separated in a 1.5% agarose gel.

ETS primers and amplification—Because universal primers for ETS are not available, we followed the general procedure of Baldwin and Markos (1998) to develop ETS primers for Neillieae. The entire intergenic spacer (IGS) of rDNA was amplified using primers IGS3 and IGS8 (Fig. 1; Appendix 2) for *Physocarpus capitatus* and primers 26S-IGS (Baldwin and Markos, 1998) and IGS88 (Fig. 1; Appendix 2) for *Aruncus dioicus* (Walter) Fernald, which was included as an exemplar for a distantly related group (Morgan et al., 1994; Potter et al., 2002). The PCR primers in the 18S gene (IGS88 and IGS8) are located ca. 300 base pairs (bp) downstream from the 5′ end of the 18S rDNA gene (Fig. 1), which allowed us to determine whether or not we had amplified the desired region by checking sequences for the presence of a portion of the highly conserved 18S gene. PCR amplifications were carried out with the Perkin-Elmer GeneAmp II kit with AmpliTaq Gold DNA polymerase (PE Biosystems) and *Taq* Extender PCR Additive (Stratagene, La Jolla, California, USA) as follows: a hot start at 95°C for 10 min; 40 cycles of denaturation at 95°C for 30 s, primer annealing at 50°C for 1 min, and primer extension at 72°C for 5 min; followed by a final extension at 72°C for 7 min. The complete IGS sequences were determined using five additional nested sequencing primers (two primers for *P. capitatus,* three for *A. dioicus*) as well as PCR primers. The nested sequencing primers are not listed in Appendix 2, but sequences for those primers are available from the first author upon request.

When the full IGS sequences from these two species were determined, a new PCR primer, ETS1, to anneal to the 3′ region of ETS (Fig. 1; Appendix 2) was developed by comparing the two IGS sequences. The ETS region was amplified using ETS1 and IGS8 primers for the species of Neillieae. PCR products purified from agarose gels were directly sequenced in both directions using the primers ETS1 and IGS6 (Fig. 1; Appendix 2). We used IGS6, which anneals at 90 bp from the 5′ end of the 18S gene, instead of IGS8, in sequencing reactions to obtain a more accurate base reading in the ETS region.

Sequence alignments—Sequences were aligned using Clustal X (Thompson et al., 1997) and adjusted manually as needed. All chloroplast sequences were concatenated to make the cpDNA data set. Because the ITS and ETS are parts of the rDNA repeat (Soltis and Soltis, 1998), sequences of these spacer regions were combined in phylogenetic analyses to make the rDNA data set. For the *LEAFY* data, all variable cloned sequences from each accession were included; however, nucleotide sequences from the two outgroup species were not included because of alignment problems.

A few sequences of cpDNA were not determined due to difficulties with the PCR or to lack of variability of nucleotide sequences across species as found in the preliminary survey, in which not all accessions in the particular species were sequenced. These sequences were treated as missing data. Of the 108 664 cells in the aligned cpDNA data matrix, 3925 (3.6%) cells were scored as missing. There were no missing cells in the rDNA and *LEAFY* data. Aligned data matrices along with phylogenetic trees were submitted to the TreeBase database (http://www.treebase.org/).

Phylogenetic analyses—Separate phylogenetic analyses for each data set were conducted employing maximum parsimony (MP) and Bayesian methods. We used PAUP* version 4.0b10 (Swofford, 2002) for the parsimony analyses. All characters were treated as unordered and weighted equally. Gaps were treated as missing data, and multiple character states at a site were interpreted as uncertainty. Heuristic searches were used in all analyses to find the MP trees with 100 replicates of random taxon addition and tree bisection–reconnection (TBR) branch swapping saving all of the best trees at each step (MulTrees). Bootstrap analyses (Felsenstein, 1985) with 500 pseudoreplicates were conducted with simple sequence addition and TBR branch swapping. No more than 1000 trees were saved for each pseudoreplication for cpDNA and rDNA data. In the case of *LEAFY* data, the "fast" bootstrap option in PAUP* (Swofford, 2002) was used with 10 000 pseudoreplicates. Bayesian phylogenetic analyses were performed with MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). A Metropolis-coupled Markov chain Monte Carlo (MCMCMC) algorithm was employed for 1 000 000 generations, sampling trees every 100 generations, with four independent chains running simultaneously. For the cpDNA and *LEAFY* data, the general time-reversal model (GTR; Swofford et al., 1996) with six rate parameters and the gamma distribution (Γ) was used, and for the rDNA data, the GTR + Γ model with two rate parameters was used to estimate the likelihood values. These evolutionary models were determined by the hierarchical likelihood ratio test using Modeltest version 3.06 (Posada and Crandall, 1998). In each analysis, all 10 001 resulting trees were imported into PAUP*, and a 50% majority-rule consensus tree was generated after discarding the first 201 trees (20 000 generations).

TABLE 2. Summary statistics of three data matrices used in our phylogenetic study of Neillieae. Values under "ingroup only" refer to the comparisons for ingroup taxa only and those under "with outgroup" indicate that statistics were calculated with outgroup sequences. Because we did not include outgroups for the analysis of the *LEAFY* data statistics of *LEAFY* are from comparison for ingroup only. Sequence divergence was calculated using Kimura's two-parameter model (Kimura, 1980) in PAUP*.

Characteristics	cpDNA		rDNA		
	Ingroup only	With outgroup	Ingroup only	With outgroup	*LEAFY*
No. of characters	3040	3196	1081	1119	2038
No. of variable characters	65	277	120	299	263
No. of phylogenetically informative characters	53	102	99	138	203
Range of sequence divergence	0–0.014	0–0.058	0–0.080	0–0.1961	0–0.147
Average sequence divergence	0.008	0.013	0.042	0.057	0.073

These "burn-in" generations, for which the log-likelihood values had not reached a plateau, were determined by plotting a graph of the log-likelihoods of each generation vs. generation numbers (Huelsenbeck and Ronquist, 2001). Topological incongruence was evaluated based upon relative bootstrap support or Bayesian posterior probability (Mason-Gamer and Kellogg, 1996). We considered topological conflicts among data partitions to be significant if discordant relationships of a given set of taxa were supported with greater than 70% bootstrap support or 95% posterior probabilities.

In combined analyses of all three data sets, we concatenated all the sequencing results from all taxa. For *LEAFY* data, we randomly selected one cloned sequence per accession or used the direct sequencing result, if available. However, in some accessions of *P. malvaceus* and *P. monogynus*, two distinct sequence types were found within an accession (see Results). For these sequences, we included two representative cloned *LEAFY* sequences per accession in the combined data set and duplicated the cpDNA and rDNA sequences.

The combined data set was analyzed employing MP, maximum likelihood (ML), and Bayesian methods. The ML analysis utilized the GTR model with six rate parameters, the proportion of invariable sites (I) = 0.5523, and the shape parameter of the gamma distribution (Γ) α = 0.7984, as determined by the hierarchical likelihood ratio test using Modeltest version 3.06 (Posada and Crandall, 1998). Heuristic searches with 100 replicates of random taxon addition, TBR branch swapping, and MulTrees options were used to find MP and ML trees with PAUP*. Reliability of each clade was evaluated by bootstrap proportions and Bayesian posterior probabilities. Bootstrap proportions for each clade were obtained only in the MP analysis, using 500 pseudoreplicates of the data with simple sequence addition, TBR branch swapping, and MulTrees options. Bayesian posterior probabilities were estimated in MrBayes. The MCMCMC algorithm was employed for 1 000 000 generations, sampling trees every 100 generations, with four independent chains running simultaneously. We applied two separate models for different partitions: GTR + Γ with six rate parameters for the cpDNA data and GTR + Γ with two rate parameters for the rDNA and reduced *LEAFY* data. The first 14 000 generations were eliminated as the "burn-in" generation, and a 50% majority-rule consensus tree was computed for the rest of trees.

Biogeographic analyses—Ancestral distributions were reconstructed from a reduced species tree from the combined analysis with the DIVA program, version 1.1 (Ronquist, 1997). This dispersal-vicariance analysis assumes that speciation is caused by vicariance and reconstructs the optimal ancestral distribution using a parsimony criterion to minimize the dispersal and extinction events. Current distribution areas for the species of Neillieae were coded in three categories (eastern Asia, eastern North America, and western North America). Because the sister relationship of Neillieae is unclear (Kalkman, 1988; Morgan et al., 1994; Potter et al., 2002), several possible combinations of outgroup distributions were explored in the reconstructions.

Estimation of divergence time—Divergence times of Neillieae were estimated by the penalized likelihood method implemented in the program r8s (Sanderson, 2002), which allows evolutionary rates to vary across a phylogeny. This semi-parametric smoothing method uses a smoothing parameter that controls rate smoothing and fitness of the data to the saturation model, in which each lineage is permitted to have a unique rate. If the smoothing parameter is set to zero it becomes the saturation model, while an extremely higher value of smoothing results in a molecular clock model, in which every lineage of a phylogeny has the same rate of change. The optimal smoothing parameter is chosen from cross-validation analysis of the data (Sanderson, 2002).

For this analysis, we generated the ML tree of Rosaceae from the combined *matK* and *trnL-trnF* sequence data in Potter et al. (2002) to estimate the age of the most recent common ancestor (MRCA) of Neillieae. The ML tree was generated in PAUP* through heuristic searches with 100 replicates of random taxon addition, TBR branch swapping, and MulTrees options. The GTR + Γ model (Swofford et al., 1996) with six rate parameters and the gamma shape parameter (α = 0.6716) was used, as determined by the hierarchical likelihood ratio test using Modeltest version 3.06 (Posada and Crandall, 1998). The ML tree with estimated branch lengths was included as the source tree in the analysis of divergence times, and the outgroups (*Rhamnus*, *Morus*, and *Ulmus*; Potter et al., 2002) were excluded before the analysis. The age of the Rosaceae was fixed at 76 million years before the present (mya) based on estimation of Wilkström et al. (2001). We used the age of fossilized *Prunus* endocarps with enclosed seeds (Middle Eocene; Cevallos-Ferriz and Stockey, 1991) to calibrate the rescaled molecular tree. It is, however, uncertain whether the fossilized fruits belong to the crown group of *Prunus* or represent stem lineages leading to the crown group. In either case, the age of the stem group (Magallón and Sanderson, 2001), i.e., the age of divergence of *Prunus* from its sister clade, Maloideae s.l., should be older than the fossil *Prunus* age. We constrained the minimum age of the MRCA of *Prunus* and its sister clade to be 44.3 mya. The optimal smoothing parameter, determined by the cross-validation procedure using the truncated Newton (TN) algorithm (Sanderson, 2002), was set to 10.

Confidence intervals of the divergence times, derived from sampling of a limited number of nucleotide characters, were estimated by the nonparametric bootstrap procedure (Baldwin and Sanderson, 1998; Sanderson and Doyle, 2001). Two hundred bootstrap trees were generated using PAUP*, enforcing the original ML topology as a constraint at each bootstrapping step. These bootstrap trees have identical topologies, but their branch lengths vary across trees because data matrices used to estimate branch lengths were bootstrapped. Branch lengths were estimated under the ML criterion using the same model described earlier. These 200 phylograms were used as source trees to estimate divergence times in r8s.

RESULTS

Sequence analyses—The exact length of each cpDNA region is uncertain because PCR primers were located in the proximal ends of coding regions and/or noncoding regions (Oh and Potter, 2003). However, ca. 2.9 kb of the cpDNA sequences were determined, and the final alignment of the cpDNA data set includes 3196 sites (Table 2).

The final alignment of rDNA data consists of 632 sites from the ITS region (ITS-1 + 5.8S + ITS-2), 427 characters from the 3′ region of ETS, and 60 sites from 18S. The unaligned length of ITS ranged from 595 to 609 bp and that of the ETS

TABLE 3. Characteristics of rDNA regions sequenced in this study. ITS refers to only the noncoding spacer regions of ITS1 and ITS2. Statistics were derived from comparisons for ingroup and both ingroup and outgroup taxa. Comparisons for ingroup taxa only are indicated in parentheses.

Characteristics	ITS	ETS	5.8S	18S
No. of characters	468 (445)	427 (412)	164 (164)	60 (60)
No. of variable characters	140 (65)	155 (54)	4 (1)	0
No. of parsimony-informative characters	70 (54)	67 (44)	1 (1)	0
% of parsimony-informative characters	15.0 (12.0)	15.7 (10.7)	0.6 (0.6)	0

region ranged from 409 to 417 bp. The partial 18S gene sequences determined were identical across Neillieae, while there were a few variations in the 5.8S gene. When noncoding portions of the ITS and ETS regions were compared, the two regions provided similar levels of sequence variation (Table 3).

The rDNA data had higher levels of sequence divergence than the cpDNA data (Table 2). The average pairwise sequence divergence of rDNA was more than four times as high as that of cpDNA with outgroup sequences, and it was more than five times as high as that of cpDNA when only ingroup sequences were compared (Table 2).

The amplification of the second intron of *LEAFY* produced a single band in agarose-gel electrophoresis except for *P. alternans* 253, from which an additional weak-intensity band was generated. All PCR products of *LEAFY* from species of Neillieae contained sequences of both exon 2 and exon 3 and intron/exon boundary sequences. The multiple alignment of the *LEAFY* sequences indicated that longer sequence from the additional faint band found in *P. alternans* 253 resulted from a unique insertion (257 bp) in the intron. With the exception of the longer sequence, the unaligned length of the second intron of *LEAFY* in *Physocarpus* ranged from 843 to 860 bp. The range of the length of the intron in *Neillia* and *Stephanandra* was from 581 to 622 bp except for the sequences from *N. thibetica*. The intron sequences from that species were about 1370 bp in length, and a 757-bp insertion was assumed in order to align the sequences with others. All of the *LEAFY* sequences from Neillieae were reliably aligned when several blocks of gaps were introduced, and the final alignment of the *LEAFY* data set consisted of 2038 sites, 57 of which were from exons.

Sequences of the second intron of *LEAFY* from outgroup species were, however, highly divergent from those of Neillieae, resulting in alignment problems (Oh and Potter, 2003). The outgroups were therefore excluded from analyses of *LEAFY* data and the trees based on *LEAFY* sequences were rooted between *Physocarpus* and *Neillia-Stephanandra*, based on the results of Potter et al. (2002) and of our analyses of the rDNA and cpDNA data in this study. The *LEAFY* data are the most variable among the three data sets (Table 2). The average pairwise sequence divergence of the *LEAFY* data among species in Neillieae was 1.7 and 9 times higher than that of the rDNA and cpDNA data, respectively.

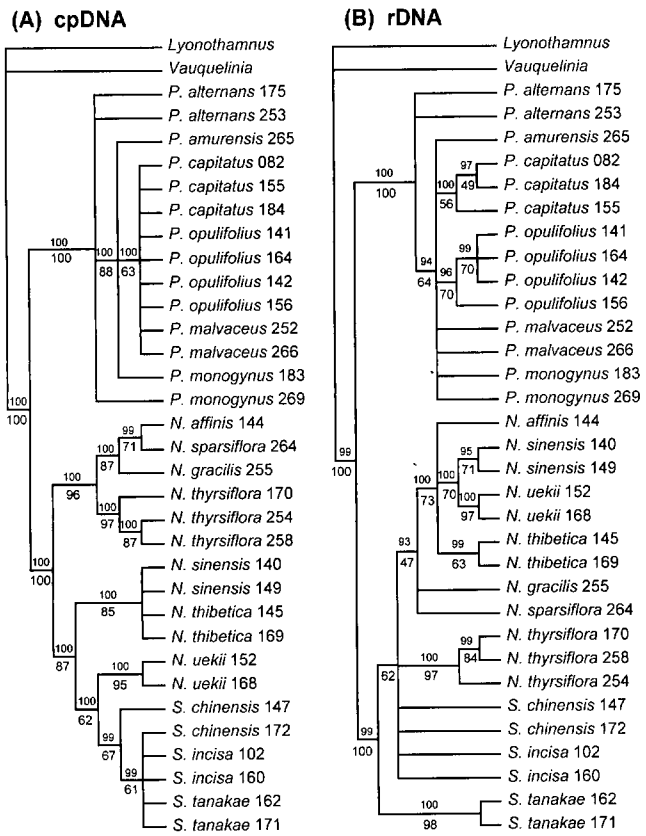

Fig. 2. Comparison of phylogenetic trees from separate analyses of the cpDNA and rDNA data. Bootstrap proportions are indicated below branches, and Bayesian posterior probabilities are shown above branches. Three-digit numbers following taxon names represent DNA accession numbers, which is used to distinguish each plant. (A) Strict consensus tree for 30 most parsimonious trees from the cpDNA data (tree length = 295, CI = 0.92, RI = 0.98). (B) Strict consensus tree for 480 most parsimonious trees based on the rDNA data (tree length = 397, CI = 0.75, RI = 0.95).

Separate phylogenetic analyses—Phylogenetic analysis of the cpDNA data set produced 30 MP trees (length = 295 steps, consistency index (CI), excluding uninformative characters = 0.92, retention index (RI) = 0.98), while 480 MP trees were found in the phylogenetic analysis of the rDNA data set (length = 397 steps, CI, excluding uninformative characters = 0.75, RI = 0.95). Both analyses revealed two strongly supported clades (*Physocarpus* and *Neillia-Stephanandra*) in Neillieae (Fig. 2). However, the relationship of *Stephanandra* with respect to *Neillia* was inconsistent between the two types of data. *Stephanandra* was supported as a monophyletic group and was nested within *Neillia* in the analysis of the cpDNA data (Fig. 2A), but this relationship was not resolved by the rDNA. While the MP analysis of rDNA data placed *S. tanakae* as sister to a weakly supported clade of *Neillia*, *S. incisa* and *S. chinensis* (Fig. 2B), the Bayesian analysis of the rDNA data suggested that both *Neillia* and *Stephanandra* are monophyletic (trees not shown). However, these alternative hypotheses of the rDNA data were poorly supported in both cases. Within the *Physocarpus* clade, the two accessions of *P. alternans* were sister to the rest of the species in *Physocarpus* in both rDNA and cpDNA analyses, but one of two accessions of *P. monogynus* (accession 269)

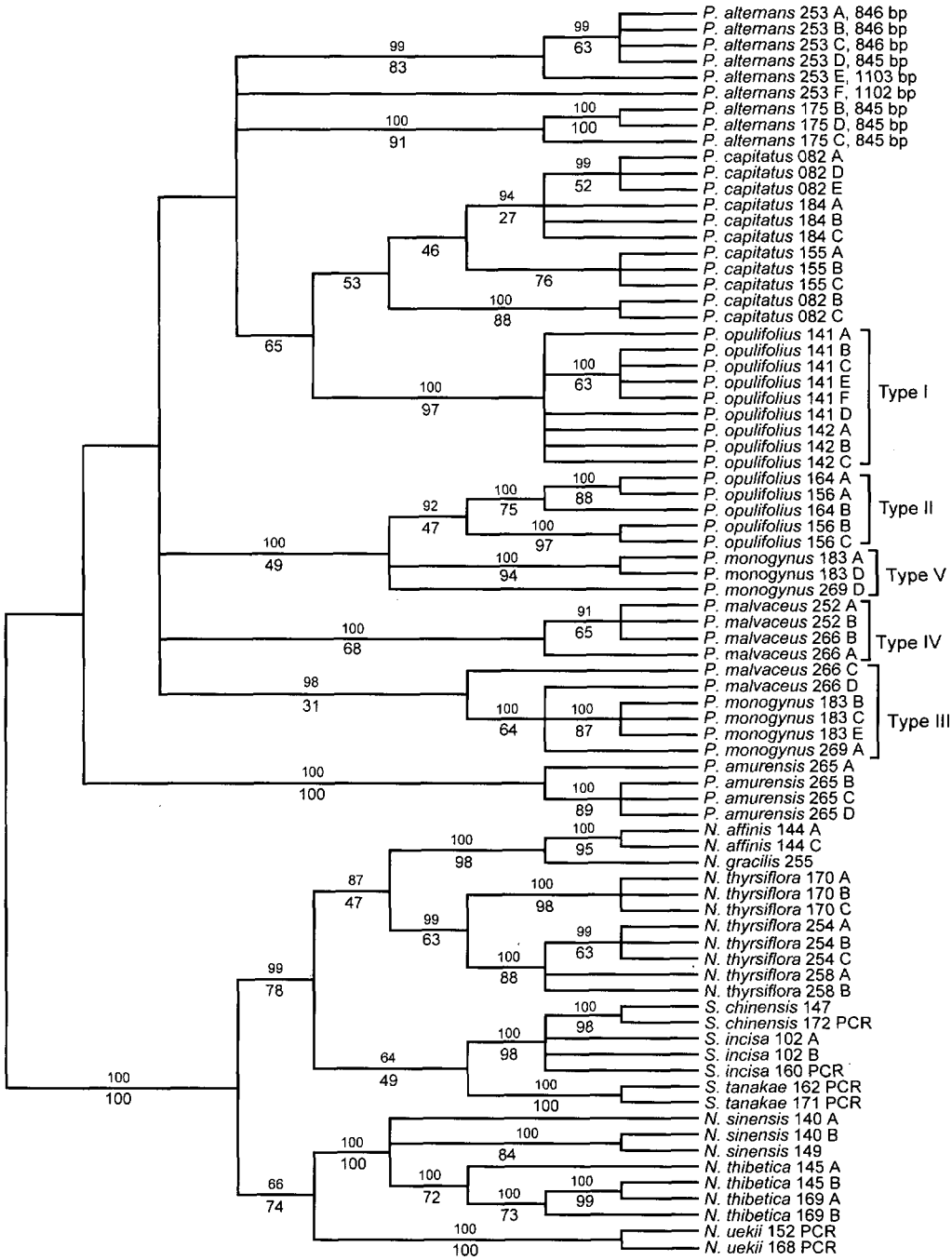

Fig. 3. Strict consensus tree for 3405 most parsimonious trees (tree length = 349, CI = 0.79, RI = 0.97), based on the *LEAFY* data. Bootstrap proportions are indicated below branches, and Bayesian posterior probabilities are shown above branches. Three-digit numbers following taxon names represent DNA accession numbers. Taxa followed by "PCR" indicate sequences determined directly from PCR products; all other sequences were determined from cloned PCR products. Length of the second intron of *LEAFY* is given in the *P. alternans* clones to indicate longer sequence only found in the accession 253. Two divergent *LEAFY* sequences are found in *P. opulifolius*; clones from accessions 141 and 142 (type I) are sister to *P. capitatus*, while sequences from accessions 156 and 164 (type II) form a clade with type V sequences of *P. monogynus* (see text).

was also placed in this position in cpDNA trees (Fig. 2A). The relationship of the eastern Asian species, *P. amurensis*, with respect to other species of *Physocarpus* was not well resolved in either analysis, but it certainly was not the first diverging lineage of *Physocarpus*.

Phylogenetic analysis of 78 cloned sequences of the second intron of *LEAFY* generated 3405 MP trees (length = 349 steps, CI, excluding uninformative characters = 0.79, RI = 0.97). In the *LEAFY* trees (Fig. 3), *Stephanandra* was supported as a monophyletic group, as in the cpDNA analysis. Unlike the cpDNA trees, in which *Stephanandra* was sister to *N. uekii*, the *LEAFY* trees placed *Stephanandra* as sister to the (*N. thyrsiflora* (*N. affinis*, *N. gracilis*)) clade (Fig. 3). The relationships among species of *Neillia* in the *LEAFY* trees are,

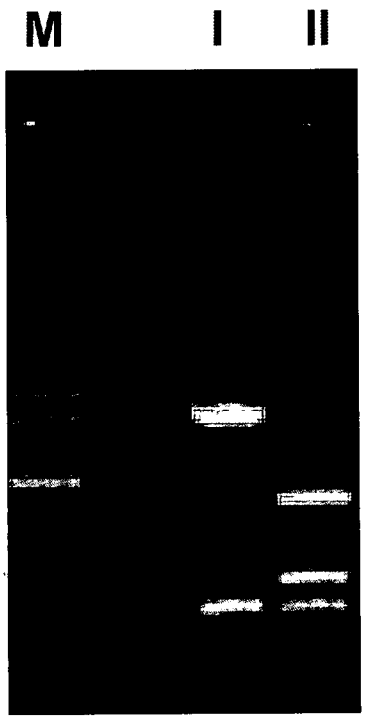

Fig. 4. Representative banding patterns of *Xba*I digestion of PCR products of the second intron of *LEAFY* from accessions of *P. opulfolius*. Lane M: 100-bp molecular marker (the brightest band represents 500 bp). Lane A: Digestion reaction for the PCR products from accession 141 (digestion of accession 142 produced the same pattern). Lane B: Digestion reaction for the PCR products from accession 156 (digestion of accession 164 produced the same pattern). The second intron of *LEAFY* from the accessions 141 and 142 has one *Xba*I site, whereas that of accessions 156 and 164 possesses two *Xba*I sites.

however, almost identical to those in cpDNA trees (Fig. 2A) when the species of *Stephanandra* are excluded.

Two divergent types of *LEAFY* sequences found in *P. opulifolius* were placed in distantly related clades not only in parsimony analysis, but also in the Bayesian analysis (Fig. 3); clones from *P. opulifolius* 141 and 142 (hereafter referred to as type I) were sister to *P. capitatus* in MP trees with moderate bootstrap support, while sequences from *P. opulifolius* 156 and 164 (hereafter referred to as type II) were sister to some sequences of *P. monogynus* in both MP and Bayesian analyses. The average sequence divergence between the types was 0.031, which is quite divergent compared to the values within types I and II (0.002 and 0.014, respectively). Only one type, not both, was cloned from each accession. The two types can be distinguished by the number of *Xba*I cleavage sites in the intron: sequences of type I have one site, while those of type II have two. *Xba*I digestion of pooled PCR products for accessions 141 and 142, from which only type I was cloned, produced two bands (lane A, Fig. 4) and the digestion for accessions of 156 and 164, from which only type II was determined, generated three bands (lane B, Fig. 4), confirming that only one type was present in each accession and that our failure to detect the second type was not an artifact of our selection of clones for sequencing.

The longer sequences in *P. alternans* 253 were either sister to the shorter sequences of this accession (clone E, Fig. 3) or formed an unresolved polytomy with other *P. alternans* sequences and the *P. capitatus-P. opulifolius* clade (clone F, Fig. 3). One accession of *P. malvaceus* (266) and both accessions of *P. monogynus* (183 and 269) contained two distinct sequence types (Fig. 3). The type III sequences (Fig. 3) from both accessions of *P. monogynus* and from *P. malvaceus* accession 266 formed a clade, as did the type IV sequences from *P. malvaceus* accession 266 and the only sequences obtained from *P. malvaceus* accession 252. The type V sequences from both accessions of *P. monogynus*, on the other hand, formed a clade with the type II sequences of *P. opulifolius*.

Because the longer sequences of *P. alternans* were found only in accession 253 and because one of them, clone E, was sister to other shorter sequences (Fig. 3), we decided to include only the shorter sequence in the combined data set. However, we included two representative cloned *LEAFY* sequences per accession in the combined data for those accessions of *P. malvaceus* and *P. monogynus* that contained two distinct sequence types.

Combined phylogenetic analyses—The parsimony analysis of the combined data produced two MP trees (length = 994 steps, CI, excluding uninformative characters = 0.77, RI = 0.96). The two trees differed only in the placement of *Stephanandra*, which was sister to either the *N. affinis-N. thyrsiflora* clade or the *N. sinensis-N. uekii* clade. One of the two MP trees (Fig. 5) was selected as the best tree ($-\ln L$ = 14444.892) in the ML analysis.

Phylogenetic relationships among species of Neillieae were fully resolved in these combined analyses except for the alternative phylogenetic positions of *Stephanandra* (Fig. 5). The combined analyses showed the well-supported sister relationship between the *Physocarpus* and *Neillia-Stephanandra* clades, as in separate analyses of rDNA and cpDNA data. The phylogenetic analyses also supported monophyly of *Stephanandra* and the placement of *Stephanandra* within *Neillia*, making the genus *Neillia* a paraphyletic group.

As in the separate analyses of cpDNA and rDNA data, the combined analyses suggested that the two accessions of *P. alternans* are the two basal-most lineages in the genus *Physocarpus* and that, among the remaining species, *P. amurensis* is sister to the rest. The four accessions of *P. opulifolius*, as in *LEAFY* data, formed two distinct clades, which are not closely related to each other, the result of divergent *LEAFY* sequences in different accessions of that species. For *P. malvaceus* 266 and both accessions of *P. monogynus*, in which each accession had two distinct *LEAFY* sequences and was therefore represented twice in the combined analysis, the results were slightly different from those of the separate analysis of the *LEAFY* data (Fig. 3). In the combined analysis, as in the separate analysis, the two sequence combinations representing *P. monogynus* accession 183 were again separated from one another as were those representing *P. malvaceus* 266; in contrast, however, the two sequence combinations representing *P. monogynus* 269 formed a clade (Fig. 5).

Biogeographic analysis—An estimated species phylogeny drawn from the combined analysis (Fig. 5) was used in the dispersal-vicariance analysis, which requires fully bifurcate trees (Ronquist, 1997). Only one terminal node per species was included in the tree with the exception of *P. opulifolius*. For *P. opulifolius*, the species was excluded in the biogeographic analysis because we suggest that it may be of hybrid origin, which violates the assumptions of DIVA (Ronquist,

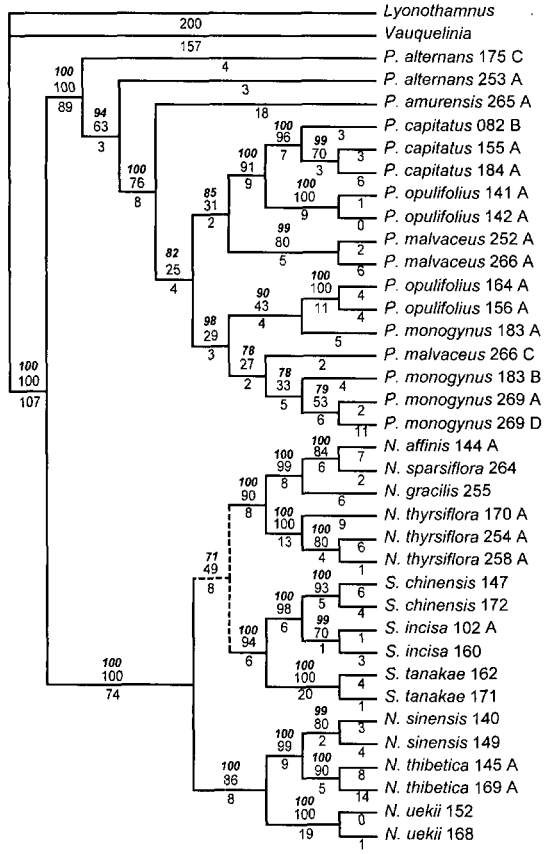

Fig. 5. One of two equally parsimonious trees (tree length = 994, CI = 0.77, RI = 0.96) and the ML tree (−ln L = 14444.892) of the combined data. Branches that collapse in the strict consensus tree in the parsimony analysis are represented by dashed lines. Bootstrap proportions and Bayesian posterior probabilities (in italics) are indicated above branches and the branch lengths (ACCTRAN optimization) in parsimony analyses are shown below branches. Three-digit numbers following taxon names represent DNA accession numbers. Taxa followed by "PCR" indicate sequences determined directly from PCR products; all other sequences were determined from cloned PCR products. Only one randomly selected cloned sequence of the second intron of *LEAFY* was included per accession with the exception of *P. monogynus* and *P. malvaceus* 266, in which two representative cloned sequences per accession were included. Nucleotide sequences of rDNA and cpDNA for the accessions were duplicated for the taxa (see text).

1997; but see Discussion for our interpretation of the origin of *P. opulifolius*).

An optimal DIVA reconstruction of the biogeographic history of Neillieae suggested ancestral distributions for the MRCA of Neillieae and that of *Physocarpus* were equivocal depending on outgroup distributions, but other internal nodes of Neillieae were constant (Fig. 6; Table 4). We explored possible areas of the MRCAs of Neillieae and *Physocarpus* for several combinations of outgroup distribution (Table 4). The results of the simulation indicated that there were two sets of optimal distributions for the MRCAs of Neillieae and *Physocarpus*: (1) the MRCA of Neillieae was distributed in eastern Asia and western North America and the MRCA of *Physocarpus* was in western North America; and (2) the MRCA of Neillieae occurred in eastern Asia and the MRCA of *Physocarpus* was distributed in eastern Asia and western North America (Table 4). In some combinations of outgroup distributions, both sets were reconstructed, but others generated

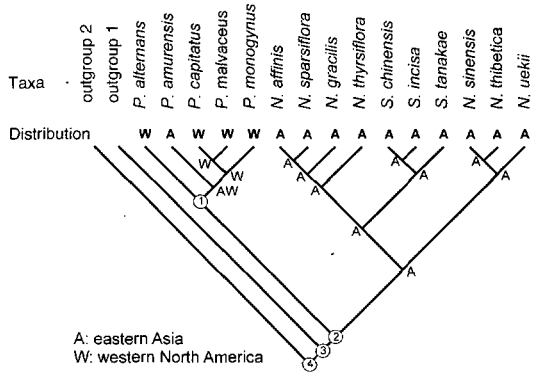

Fig. 6. Optimal reconstruction of the ancestral distributions of Neillieae using the program DIVA. The ancestral distributions not affected by outgroup distributions are given at ancestral nodes. Numbered nodes are ambiguous depending on the outgroup distribution; the inferred possible distributions of the nodes are listed in Table 4. Current distributions are indicated at terminal nodes, below taxon names.

only the second set of distributions for the MRCAs of Neillieae and *Physocarpus*. Five dispersals were required in the reconstruction if both outgroups were distributed in all areas, and two dispersals were necessary in other reconstructions in which each outgroup was assumed to occupy only one area (Table 4). No extinction was required in any of the reconstructions.

Estimation of divergence times—The single ML tree (−ln L = 16893.161) generated from the combined *matK* and *trnL-trnF* data was identical to one of the MP trees (topology "A") in Potter et al. (2002). The age of the MRCA of Neillieae with a 95% confidence interval was estimated to be 20.6 ± 0.4 mya, while that of the *Neillia-Stephanandra* clade was 3.8 ± 0.2.

TABLE 4. Reconstructed distributions of the four nodes numbered in Fig. 5. Node 1 = the MRCA of *Physocarpus*; node 2 = the MRCA of Neillieae; node 3 = outgroup node 1; node 4 = outgroup node 2. A = eastern Asia; E = eastern North America; W = western North America. No extinction was required in any of the reconstructions.

Outgroup distribution		Reconstructed distribution of nodes				No. of dispersal events
Outgroup 1	Outgroup 2	1	2	3	4	
AEW	AEW	W	AW	W	AEW	5
			or			
		AW	A	A	AEW	
A	A	W	AW	A	A	2
			or			
		AW	A	A	A	
E	E	W	AW	AEW	AEW	2
W	W	W	AW	W	W	2
A	E	AW	A	A	AE	2
			or			
		W	AW	AW	AEW	
			or			
		W	AW	A	AE	
E	W	AW	A	AE	AEW	2
			or			
		W	AW	AE	AEW	
			or			
		W	AW	AEW	AEW	
W	A	W	AW	W	AW	2

DISCUSSION

Phylogenetic utility of different genes—This study demonstrates that an intron of a single-copy nuclear gene, *LEAFY*, is the most variable and that the cpDNA data have the lowest variability of the three data sets obtained in Neillieae (Table 2). This pattern of sequence variability generally agrees with previous studies of other groups (e.g., Small et al., 1998; Bailey and Doyle, 1999). In terms of levels of homoplasy as measured by CI and RI, the cpDNA data provide the fewest character conflicts of the three data sets, and the *LEAFY* data have slightly less homoplasy than rDNA. Phylogenetic utility of the second intron of *LEAFY* was discussed in Oh and Potter (2003).

We do not present separate phylogenetic analyses of ITS and ETS regions in this paper because both regions are part of the rDNA repeat (Soltis and Soltis, 1998), and separate analyses of the two regions generated trees with topologies similar to those based on combined rDNA data (Fig. 2B). Previous studies using the ETS region, especially of Asteraceae (Baldwin and Markos, 1998; Linder et al., 2000; Markos and Baldwin, 2001), have shown that ETS has a higher proportion of phylogenetically informative characters than ITS does. Unlike the previous reports, our study indicates that the ETS region provides a lower percentage of parsimony-informative characters than ITS (Table 3). Combining the two regions, however, improves resolution and increases bootstrap support for clades, which agrees with previous reports (Baldwin and Markos, 1998; Bena et al., 1998; Markos and Baldwin, 2001).

As judged by clade supports in our separate analyses of three data sets, there are some strong topological conflicts among data partitions. For example, our cpDNA data strongly conflict with the *LEAFY* data in terms of the placement of *Stephanandra*, and the rDNA data are incongruent with the cpDNA and *LEAFY* data with respect to the relationship of *N. affinis*. Potential causes of the conflicting relationships among gene trees are discussed in the next two sections, and should better be explained when more data, especially from additional nuclear genes, are collected.

Phylogeny of Physocarpus—Phylogenetic analyses based on rDNA and combined data suggest that *P. alternans* is sister to the rest of the species of *Physocarpus* (Figs. 2B, 5). This species, which occurs in desert mountains of western North America, is morphologically distinct in the genus (Howell, 1931; Rosatti, 1993). Unlike other *Physocarpus* species, which have two or three to five carpels, *P. alternans* usually has only one carpel, which is a common characteristic in *Neillia* and *Stephanandra*. The carpel number character may support the placement of *P. alternans* as the basal lineage in the genus if the single carpel is a synapomorphy for Neillieae and the 2–5-carpel condition evolved in *Physocarpus*. It is possible, however, that the unicarpellate conditions in *P. alternans* and the *Neillia-Stephanandra* clade evolved independently. Because of lack of resolution regarding outgroup relationships of Neillieae in the Rosaceae (Potter et al., 2002), it is difficult to establish the polarity of this character in the tribe.

The *LEAFY* data, on the other hand, place *P. amurensis* as sister to the other *Physocarpus* species and *P. alternans* as sister to *P. capitatus* and *P. opulifolius* (Fig. 3). These relationships, however, are not supported in the bootstrap analysis or in the Bayesian analysis. *Physocarpus amurensis*, an eastern Asian species, was previously considered to be closely related to *P. opulifolius* and *P. capitatus* (Rehder, 1940; Robertson, 1974) in having three to five carpels that are united at the base. Our molecular data, however, do not support a close relationship between *P. amurensis* and either *P. opulifolius* or *P. capitatus* (Figs. 2, 3, 5). Morphologically, other characteristics in the fruits of *P. amurensis* differ from those of *P. opulifolius* and *P. capitatus*. The follicles of *P. amurensis* are not highly inflated at maturity and are slightly longer than or as long as the hypanthium and the sepals (Maximowicz, 1859; Poyarkova, 1939), whereas those of *P. opulifolius* and *P. capitatus* are highly inflated and are more than twice as long as the hypanthium and the sepals. In addition, our close examination of herbarium specimens of *P. amurensis*, including the possible isotype of the species, indicates that *P. amurensis* has two, rarely three, carpels, not three to five carpels. We therefore favor the hypothesis of relationships depicted in Fig. 5, in which *P. alternans*, with one carpel, and *P. amurensis*, with two to three carpels, are successive sisters to the remaining (2– or 3–5-carpellate) species of *Physocarpus*.

Our molecular data show that *P. opulifolius*, *P. capitatus*, and *P. malvaceus* are closely related, but they are separable. In the cpDNA tree, accessions of the three species form a clade with 63% bootstrap support and Bayesian posterior probability of 100 (Fig. 2A), but there is no resolution among the species. All four accessions of *P. opulifolius*, however, share a unique 2-bp indel in the *psbA-trnK* region of cpDNA. This indel character, not scored as a separate character, is the only difference in the cpDNA data, but it suggests that *P. opulifolius* is distinct from *P. capitatus* and *P. malvaceus*. Accessions of *P. opulifolius* and *P. capitatus* form separate clades in rDNA trees (Fig. 2B), while relationships among the accessions of *P. malvaceus* are unresolved. Morphologically, *P. malvaceus* can be easily distinguished from other two species by having two (vs. 3–5) carpels, which develop into flattened follicles at maturity, while *P. capitatus* can be distinguished from *P. opulifolius* in having leaves of the flowering branches that are ovate with truncate to cordate bases and marginal teeth that are acute or acuminate.

The relationships among the *LEAFY* sequences in *P. monogynus* and *P. malvaceus* are complex. Two distinct *LEAFY* sequence types are found in both accessions of *P. monogynus* and one accession of *P. malvaceus* (Fig. 3). The influence of these different *LEAFY* sequence types was evident in the combined analysis (Fig. 5), in which the different sequence combinations for an accession were separated from one another in *P. monogynus* 183 and *P. malvaceus* 266.

Due to lack of phylogenetic resolution, it is unclear what causes the complex pattern of phylogenetic relationships of these *LEAFY* sequences. It is possible that gene duplication occurred on the stem lineage of the MRCA of *P. monogynus* and *P. malvaceus*, if the two species are sister taxa, as is suggested by the placement of some of the sequence combinations in Fig. 5. Gene flow or allelic variation or a combination of both may also result in the relationships observed in the *LEAFY* data. Doyle (1995) argued that gene tree topologies might not agree with genealogical relationships among individuals or species, in part because some alleles found in a species are more closely related to alleles in other species than to alleles in the same species.

The situation is even more complex if one considers the cpDNA data. Our results indicate that two distinct cpDNA types occur within *P. monogynus* because the two accessions of that species did not form a monophyletic group in the phy-

logenetic analysis of the cpDNA data (Fig. 2A). Accession 269 collected in the Sandia Mountains in New Mexico had the same substitution pattern as *P. alternans* and shared a 22-bp insertion in the *trnL-trnF* region with *P. alternans* 253. Accession 183 from Colorado collapsed in an unresolved trichotomy with *P. amurensis* and the *P. capitatus-P. malvaceus-P. opulifolius* clade (Fig. 2A), but it had a different cpDNA profile from *P. amurensis*. We determined the cpDNA regions from two additional plants from the Sandia Mountain area, and they were identical to those from accession 269. Neither of the two nuclear DNA markers (rDNA and *LEAFY*) supports a close relationship between *P. monogynus* 269 and *P. alternans* (Figs. 2A, 3), and examination of the voucher specimens of the two *P. monogynus* accessions did not yield any evidence that *P. monogynus* 269 was more closely related to *P. alternans* than it is to *P. monogynus* 183. The presence of two cpDNA haplotypes in *P. monogynus* (Fig. 2A), along with lack of differentiation in nuclear markers and morphology, suggests that the population from the Sandia Mountains may have maintained the ancestral cpDNA haplotype shared with some of *P. alternans* populations, known as lineage sorting (Doyle, 1992) or deep coalescence (Maddison, 1997), or that they may have been derived from introgressive hybridization between *P. alternans* and *P. monogynus*, as has been shown in other plant groups (e.g., Soltis et al., 1991; Wolfe and Elisens, 1995). More extensive sampling of individuals and populations of those species with multiple data sets may help to resolve this issue.

The *LEAFY* data provide interesting implications for the origin of *P. opulifolius*. Phylogenetic analysis of the *LEAFY* data show that two divergent types of sequences, placed in distantly related clades, exist in *P. opulifolius* (Fig. 3). These two groupings are not associated with geographic distribution. For example, both accessions 142 and 156 were collected from North Carolina (Appendix 1); however, sequences from these accessions were placed in distantly related clades.

The presence of more than one type of sequence could result from allelic variation, gene duplications/losses, lineage sorting of ancestral polymorphisms, and/or hybridization/introgression (Doyle, 1992, 1995; Maddison, 1997). We hypothesize that the two distinct types of *LEAFY* sequences in *P. opulifolius* represent homeologous genes that were derived by hybridization between *P. capitatus* and *P. monogynus*, with type I derived from a gene contributed by *P. capitatus* or its ancestor and type II derived from a gene contributed by *P. monogynus* or its ancestor; the latter would also have given rise to the type V sequences of *P. monogynus* in our analysis. If the two types of sequence were paralogues resulting from gene duplication, both types of sequences (types I and II) should be present in each individual of the species. If the two types of sequences were homeologous alleles, as we propose, they would show a segregation pattern depending on the heterozygosity of a particular individual. Our *LEAFY* data show that only one type of sequence is present in one accession for all four accessions examined (Figs. 3, 4). This does not necessarily mean that the putative homeologous *LEAFY* alleles are fixed in all accessions of *P. opulifolius*; we examined two cultivated individuals of *P. opulifolius* that contain both types of sequences (types I and II) in each individual verified by a phylogenetic analysis and *Xba*I digestion (data not shown). We, however, excluded the two accessions because the geographic origin of the samples was unknown.

In summary, lineage sorting and interspecific gene flow may all have been important in the evolution of North American *Physocarpus*. Accessions of *P. monogynus* and *P. malvaceus* share alleles of *LEAFY* that have closer relationships between than within species, while the Sandia Mountain population of *P. monogynus* harbors a cpDNA type identical to that of some *P. alternans*. Finally, it appears that *P. opulifolius* was derived by hybridization between *P. monogynus* and *P. capitatus*, or their respective ancestors.

Phylogeny of the Neillia and Stephanandra clade—Phylogenetic relationships in the *Neillia-Stephanandra* clade are relatively well resolved compared to those in *Physocarpus* (Figs. 2, 3, 5). Our molecular data suggest that *N. sinensis* and *N. thibetica* are closely associated with *N. uekii*. A possible morphological synapomorphy supporting a close relationship of the three species is the short petiole (less than 1 cm long); other species of *Neillia*, *S. tanakae*, and *Physocarpus* have petioles longer than 1 cm.

Neillia sinensis and *N. thibetica* together form a clade with strong support in separate analyses of the cpDNA and *LEAFY* data and the combined analyses (Figs. 2A, 3, 5), whereas the rDNA data suggest that *N. sinensis* is sister to *N. uekii* (Fig. 2B). *Neillia sinensis*, widely distributed in China, is morphologically very similar to *N. thibetica*; both species have racemes of pink flowers with long cylindrical hypanthia (Cullen, 1971; Yu and Ku, 1974). *Neillia uekii*, endemic to Korea and northeastern China, also has racemose inflorescences, but the flowers are in creamy colors with campanulate hypanthia. The inflorescence rachis of *N. uekii* is pubescent with stellate trichomes, whereas those of *N. sinensis* and *N. thibetica* are glabrous or pubescent with simple unicellular trichomes.

Our separate analyses of cpDNA and *LEAFY* data suggest that *N. affinis*, *N. gracilis*, and *N. sparsiflora* (the latter was not represented in the *LEAFY* data because we were unable to amplify the region from the DNA isolated from an herbarium specimen), all of which are distributed in western China, form a well-supported clade (Figs. 2A, 3). This relationship is also strongly supported in our combined analyses (Fig. 5). One potential synapomorphy of the three species is the aggregation of flowers at the apex of the inflorescence, although some variation can be found in *N. affinis*. *Neillia sparsiflora* and *N. gracilis* are morphologically very distinct in the genus (Cullen, 1971). *Neillia sparsiflora* is characterized by having capitate-glandular trichomes on the flowering branches, veins of the lower leaf surfaces, petioles, stipule margins, bracts, and inflorescence, while plants of *N. gracilis* are suffrutescent and rhizomatous, reaching only 0.5 m in height. Interestingly, *N. affinis* is placed in a well-supported clade with *N. sinensis*, *N. thibetica*, and *N. uekii* by our rDNA data (Fig. 2B). More data are necessary to reconcile these discordant results.

Both cpDNA and *LEAFY* data, separately and in combination, support *N. thyrsiflora* as sister to the *N. affinis-N. gracilis* clade, although the rDNA data alone do not resolve the relationship (Figs. 2, 3, 5). There are a few morphological characters that can define the clade. The paniculate inflorescence is a possible synapomorphy, but it would have to have been lost in *N. sparsiflora*.

The genus *Stephanandra* consists of only three eastern Asian species. Species of *Stephanandra* form a monophyletic group, being nested within *Neillia* in both cpDNA and *LEAFY* trees, while the rDNA data do not provide any consistent relationship with confidence. The topologies of the cpDNA and *LEAFY* trees are nearly identical when *Stephanandra* is ex-

cluded; only the phylogenetic placement of *Stephanandra* is different between the two trees (Figs. 2A, 3). Oh and Potter (2003) have postulated that *Stephanandra* may have originated via hybridization between two lineages of *Neillia* (*N. uekii* and the *N. affinis-N. thyrsiflora* clade), although other processes, such as gene duplication/loss and lineage sorting, cannot be ruled out as causes of the incongruent positions of *Stephanandra* supported by the two data sets. Under the hybridization model, the ovulate progenitor of *Stephanandra* may have been *N. uekii* or its recent ancestor because the cpDNA tree, which reflects the maternal phylogeny of the two genera, places *Stephanandra* as sister to *N. uekii* (Fig. 2A). On the other hand, *LEAFY* trees, representing nuclear gene trees, do not support the sister relationship between *Stephanandra* and *N. uekii*, but place *Stephanandra* as sister to the *N. affinis-N. thyrsiflora* clade (Fig. 3), which suggests that the stem lineage leading to this clade might have been the pollen progenitor of *Stephanandra*. Oh and Potter (2003) have further suggested that the paternal homeologous allele of *LEAFY* in *Stephanandra* was fixed, resulting in the disparity between cpDNA and *LEAFY* trees. It is difficult to draw conclusions regarding this hybrid hypothesis from the rDNA data because the phylogenetic relationship between *Neillia* and *Stephanandra* is poorly resolved (Fig. 2B).

We propose that *Neillia* and *Stephanandra* should be merged into one genus, in which case the name *Neillia* should be used because it has priority over *Stephanandra*. Our analyses of cpDNA and *LEAFY* data (Figs. 2A, 3) and the combined analysis (Fig. 5) suggest that *Stephanandra* is nested within *Neillia*, while rDNA data alone did not resolve the relationship. The two genera, however, form a strongly supported monophyletic group in all analyses. The close relationship between *Neillia* and *Stephanandra* is also supported by morphological characteristics, such as the acuminate to caudate leaf apex, racemose or paniculate inflorescence, and possession of a single carpel.

Biogeographic history of Neillieae—The DIVA reconstructions indicate that the ancestral distributions for the MRCA of Neillieae (node 2; Fig. 6) and the MRCA of *Physocarpus* (node 1; Fig. 6) are ambiguous depending on outgroup distribution (Table 4). In all cases, however, the MRCAs of Neillieae and *Physocarpus* are not distributed in eastern North America, even if one or both outgroups are assumed to have occupied the area (Fig. 6; Table 4). The biogeographic analysis of Neillieae suggests that species of *Neillia* and *Stephanandra* evolved in eastern Asia and diversified in the same area (Fig. 6; Table 4).

There are two optimal biogeographic scenarios for *Physocarpus* suggested by DIVA depending on outgroup distribution (Fig. 7; Table 4). If the MRCA of Neillieae was widely distributed in both western North America and eastern Asia, the MRCA of *Physocarpus* originated in western North America (Fig. 7A). In this case, vicariance resulted in the split of *Physocarpus* and the *Neillia-Stephanandra* clade and one dispersal event to eastern Asia must be assumed at an early stage during the evolution of *Physocarpus*. The other optimization, in which the MRCA of Neillieae was distributed only in eastern Asia, requires two independent dispersal events to western North America from eastern Asia during the evolution of *Physocarpus* (Fig. 7B). The former biogeographic scenario is more likely because it requires fewer dispersal events in the *Physocarpus* lineage than the latter hypothesis and is consistent with other reconstructions with certain combinations of outgroup distribution (e.g., A, W; E, E; or W, W; Table 4).

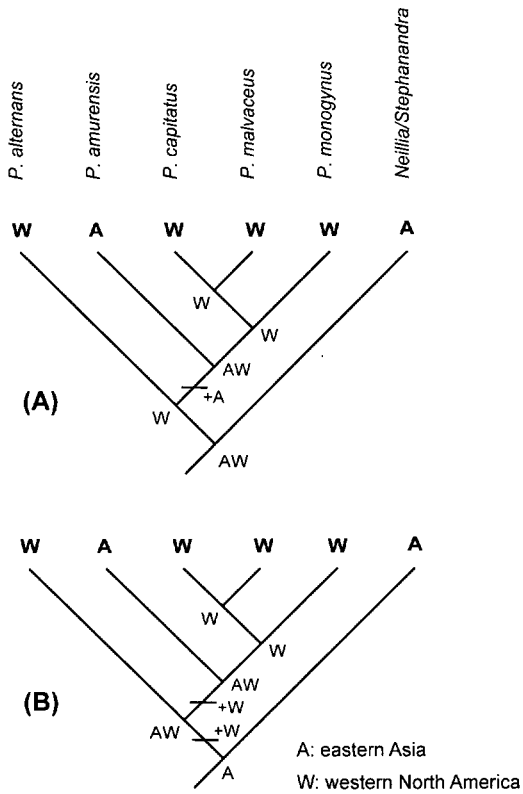

Fig. 7. Suggested biogeographic history of Neillieae with reduced terminals in the *Neillia-Stephanandra* clade based on two optimal reconstructions with the program DIVA. Dispersal events outside of the most recent common ancestor (MRCA) of Neillieae are not shown. (A) The MRCA of Neillieae was widely distributed in eastern Asia and western North America. (B) The MRCA of Neillieae was distributed only in eastern Asia.

If the MRCA of Neillieae would have been widely distributed in both western North America and eastern Asia as suggested by DIVA analysis (Fig. 7A), the continuous distribution of the ancestral population might have been achieved through the Bering land bridge. The age of the MRCA of the Neillieae is estimated to be the early Miocene, at which time the land bridge physically connected the two continents and the environment was favorable for temperate deciduous shrubs, such as species of Neillieae, to spread via the land bridge (Tiffney and Manchester, 2001). It is less likely that the North Atlantic land bridge would be involved in the ancestral distribution because it was rarely available for a dispersal pathway in the Miocene and because there is no fossil evidence for Neillieae having been native to Europe.

Our age estimation indicates that extant species of *Neillia* and *Stephanandra* might have radiated after the Pleiocene in eastern Asia. However, the timing of the biogeographic event within *Physocarpus* involving western North America and eastern Asia (Fig. 7A) is unknown because divergence times were estimated based upon a family-level phylogeny, in which *Physocarpus* is represented by only one species. It would be crucial to estimate node ages within *Physocarpus* using a species-level phylogeny, such as our combined tree (Fig. 5), but uncertainty regarding the placement of *Physocarpus* fossils to the current phylogeny makes it difficult to calibrate the mo-

lecular tree. It certainly should not predate the first vicariance event at the MRCA of Neillieae. In other words, it must have happened after the Miocene via the Bering land bridge, which was periodically available from the Early Tertiary until the present (Donoghue et al., 2001; Tiffney and Manchester, 2001). Therefore, biogeographic episodes, involving continuous distribution of ancestral populations across eastern Asia and western North America followed by vicariance, should have happened twice during the evolution of Neillieae (Fig. 7A). Our findings agree with Donoghue et al. (2001), in which a series of repeated vicariance and dispersal events between eastern Asia and North America through Beringia at different times in different clades have resulted in the current distribution patterns in those amphi-Pacific, bicontinental disjunct groups. However, we do not find any evidence that may support a scenario (Donoghue et al., 2001) involving an initial diversification within Asia before the intercontinental migration and vicariance in Neillieae; our results therefore support the view that the biogeographic history of the Northern Hemisphere is complex and heterogeneous (Tiffney, 1985a, b; Wen, 1999; Xiang et al., 2000; Xiang and Soltis, 2001).

Fossils—Fossils of *Physocarpus* have been reported from North America, whereas no fossil evidence for *Neillia* and *Stephanandra* is available. As discussed previously, those fossil records are not informative enough to be used to date the molecular tree, however. Two megafossil species of *Physocarpus*, dated to 26.5 mya (Late Oligocene), were described from southern Colorado (Axelrod, 1987). They are considered to be closely related to the extant species *P. capitatus* and *P. opulifolius* (Axelrod, 1987); both are currently not distributed in the area. However, the two fossil species were later reclassified as *Ribes* in Grossulariaceae (Wolfe and Schorn, 1990), with which we agree.

Fossilized leaves from the middle Eocene chert in Republic have been assigned to *Physocarpus* (Wehr and Hopkins, 1994) and to an extinct group closely related to *Physocarpus* (Wolfe and Wehr, 1991; Wehr and Hopkins, 1994). It is often difficult to identify fossil leaves of Rosaceae to genus without flowers and/or fruits. In particular, those fossilized leaves assigned to *Physocarpus* might in fact represent species of, or closely related to, *Rubus* or *Crataegus* (or even, as noted above, *Ribes*). One unique leaf character of extant *Physocarpus* species in Rosaceae is the distribution of stellate trichomes. None of the fossilized leaves from Republic has stellate trichomes, however. The fossils are much older than the age of the MRCA of Neillieae based upon a molecular phylogeny of Rosaceae (middle Eocene vs. early Miocene), implying that they are not closely related to modern *Physocarpus* and that they perhaps belong to the stem lineage of Neillieae. In addition, our knowledge of evolutionary patterns (of, e.g., leaf architectural characters) among the major clades of Rosaceae is far from complete, which sometimes makes it difficult to place fossilized leaves onto a phylogeny of extant members.

Other megafossils of *Physocarpus* have been reported from four localities (all ca. 3 mya) in the Canadian Arctic Archipelago (Matthews and Ovenden, 1990). The taxonomic affinities of these fossils to extant species are unknown, however.

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