Molecular Systematic Analysis Reveals Cryptic Tertiary Diversification of a Widespread Tropical Rain Forest Tree

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ABSTRACT: The broad geographic range of many Neotropical rain forest tree species implies excellent dispersal abilities or range establishment that preceded the formation of current dispersal barriers. In order to initiate historical analyses of such widespread Neotropical trees, we sequenced the nuclear ribosomal spacer (ITS) region of *Symphonia globulifera* L. f. (Clusiaceae) from populations spanning the Neotropics and western Africa. This rain forest tree has left unmistakable Miocene fossils in Mesoamerica (15.5–18.2 Ma) and in South America (~15 Ma). Although marine dispersal of *S. globulifera* is considered improbable, our study establishes three marine dispersal events leading to the colonization of Mesoamerica, the Amazon basin, and the West Indies, thus supporting the paleontological data. Our phylogeographic analysis revealed the spatial extent of the three Neotropical *S. globulifera* clades, which represent trans-Andes (Mesoamerica + west Ecuador), cis-Andes (Amazonia + Guiana), and the West Indies. Strong phylogeographic structure found among trans-Andean populations of *S. globulifera* stands in contrast to an absence of ITS nucleotide variation across the Amazon basin and indicates profound regional differences in the demographic history of this rain forest tree. Drawing from these results, we provide a historical biogeographic hypothesis to account for differences in the patterns of β diversity within Mesoamerican and Amazonian forests.

Keywords: internal transcribed spacer (ITS), phylogeography, molecular clock, fossils, long-distance dispersal, tropical trees.

Many prominent rain forest trees are broadly distributed in the American tropics (Pitman et al. 1999), with conspecific populations in Mesoamerica, Amazonia, the West Indies, and the Atlantic Forests of Brazil. Formidable geographic barriers to gene flow—including mountain chains, oceans, and xeric habitats—surround the main tracts of Neotropical rain forest, suggesting that widespread rain forest plants have superior dispersal abilities, were established before the formation of contemporary barriers, or both. A strict dispersal scenario suggests that many species should mix over broad areas, thus contributing to the high α but relatively low β diversity of rain forest trees recently observed in western Amazonia (Pitman et al. 1999; Condit et al. 2002). A vicariance history, however, would imply that the morphological uniformity of widespread species belies cryptic evolutionary divergence among their populations. In support of this idea, Raven (1999) hypothesized that the large number of lowland rain forest plant species occurring east and west of the Andes in Ecuador (1,431 species; Jørgensen and León-Yánez 1999) provides evidence that approximately 30% of the rain forest flora evolved before the rise of the Andes, setting a minimum age of several million years for conspecific cross-Andean populations.

Although the importance of ecological processes in shaping the distribution of species diversity in tropical tree communities is undisputed (Hubbell 2001; Wright 2002), biogeographic history must contribute strongly to observed patterns through its influence on regional differences in speciation, extinction, and immigration (Ricklefs and Schluter 1993). The historical contribution can be evaluated with a paleontological or phylogenetic approach. Morley (2000) combined fossil evidence with plate tectonic reconstructions to infer the origin and dispersal history of tropical rain forests since the mid-Cretaceous rise of angiosperms. Other authors have calibrated molecular phylogenies with fossil dates to trace the diversification and dispersal history of such prominent rain forest plant families as the Melastomataceae (Renner et al. 2001) and Malphigiaceae (Davis et al. 2002). Parallel studies may be performed at lower taxonomic levels. Under the assumptions of Hubbell's (2001) neutral model, common and widespread tropical rain forest tree species should have an an-

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cient history owing to the large number of generations that are required to occupy ecological space in species-rich forests. If geographically isolated rain forest trees have independent evolutionary histories, then phylogenetic approaches could be used to garner insights into the biogeographic history of species and forests over the timescales in which communities are assembled. Unfortunately, relatively few Neotropical studies of trees have sampled across political or biogeographic boundaries (Aide and Rivera 1998; Gillies et al. 1999) and, before this study, there had been no phylogeographic analyses of Neotropical tree populations based on DNA sequence data.

To initiate phylogeographic investigations of widespread Neotropical plants, we studied the rain forest tree *Symphonia globulifera* L. f. (Clusiaceae), which occurs throughout the Neotropics and tropical Africa and whose regional history is provided in broad outline by fossil records. The first pollen fossils of *Symphonia* appear in Africa at ~45 Ma (Jan-du-Chene et al. 1978). The first Mesoamerican fossils of *S. globulifera* appear in the early/middle Miocene (15.5–18.2 Ma; Fournier 1982) and in South America by the mid-Miocene (~15 Ma; Germeraad et al. 1968). Thus, this taxon has inhabited Central and South American rain forests for at least 15 million years. To elucidate the biogeographic history of *S. globulifera*, we analyzed DNA sequences representing the nuclear ribosomal spacers (ITS, internal transcribed spacer of 18S-26S nuclear ribosomal DNA) from populations sampled throughout the Neotropics. The deep divergence we found among *S. globulifera* lineages illustrates an evolutionary history that is considerably more complex than implied by the simple taxonomy of this species, renowned for its morphological uniformity. Moreover, the strong signal of regional history in the phylogenetic analysis of *Symphonia* indicates that community-level analysis of tropical forests should include careful consideration of regional biogeography in addition to contemporary ecological processes.

Material and Methods

Study Species

Symphonia globulifera is morphologically distinctive, with aerial roots and a bright yellow resin that distinguish it in species-rich Neotropical forests (fig. A1 in the online edition of the *American Naturalist*). *Symphonia globulifera* is found along rivers and in terra firme rain forests in tropical Africa and the Neotropics including the West Indies. It is the only recognized species of its genus outside of Madagascar, which harbors 16 *Symphonia* species. The intricate scarlet flowers of *S. globulifera* are visited primarily by hummingbirds and perching nectar-feeding birds (Bittrich and Amaral 1996; Gill et al. 1998). *Symphonia globulifera* produces ~3-cm berries that are consumed by ruminants (in Africa), large birds, monkeys (Gautier-Hion et al. 1985), and bats (Aldrich and Hamrick 1998). Although the seeds survive for short periods in fresh water (Scarano et al. 1997), they lack dormancy and die quickly with desiccation (Maury-Lechon et al. 1980), thus raising the question of how the species achieved its trans-Atlantic distribution. The species is of pharmaceutical interest, since resin in root samples from the Central African Republic has been shown to contain secondary compounds that inhibit replication of the human immunodeficiency virus (Gustafson et al. 1992).

Symphonia has very distinctive pollen and pollen fossils, and the latter is used by the oil industry for stratigraphic dating (R. J. Morley, personal communication). The fossil pollen taxon representing *Symphonia* is named *Pachydermites diederexi*. The earliest records of *P. diederexi* are from the mid-Eocene (~45 Ma) of Nigeria (Jan-du-Chene et al. 1978), and there are unpublished records from Angola through the Oligocene and Miocene (R. J. Morley, personal communication). *Pachydermites diederexi* occurs in early/mid-Miocene (15.5–18.2 Ma; Fournier 1982) and mid-Pliocene (~4 Ma) sediments in Mexico (Graham 1976; A. Graham, personal communication), in the Plio-Pleistocene of southeast Costa Rica (Graham and Dilcher 1998), and in mid-Miocene deposits (~15 Ma) in the Maracaibo basin of Venezuela (Germeraad et al. 1968). There are no fossil records from North America, Eurasia, East Africa, or Madagascar (R. J. Morley, personal communication). Since there are no other *Symphonia* species in the New World, we assume that the Neotropical fossil record of *P. diederexi* refers specifically to *S. globulifera*.

Madagascar is considered the geographic origin of the genus *Symphonia* by most authors because it harbors most of the species diversity (Germeraad et al. 1968). Although Desjardin et al. (1973) have proposed a Neotropical origin of *Symphonia*, the geographic and temporal distribution of *P. diederexi* presented here favors an African origin.

Field Collections

We collected fresh leaves of *S. globulifera* from upland forests and permanent inventory plots in Costa Rica, Panama, Ecuador, Brazil, French Guiana, Bolivia, and Cameroon (fig. 1). In the plots, each tree is georeferenced and permanently marked with a numbered aluminum tag. We supplemented our geographic coverage of *S. globulifera* with DNA extracted from herbarium accessions representing sites in western Panama, Belize, and Dominica (Lesser Antilles; table B1 in the online edition of the *American Naturalist*). To assess local variation in the ITS haplotypes, we sequenced up to 18 individuals per site in the 50-ha inventory plots managed by the Center for Tropical

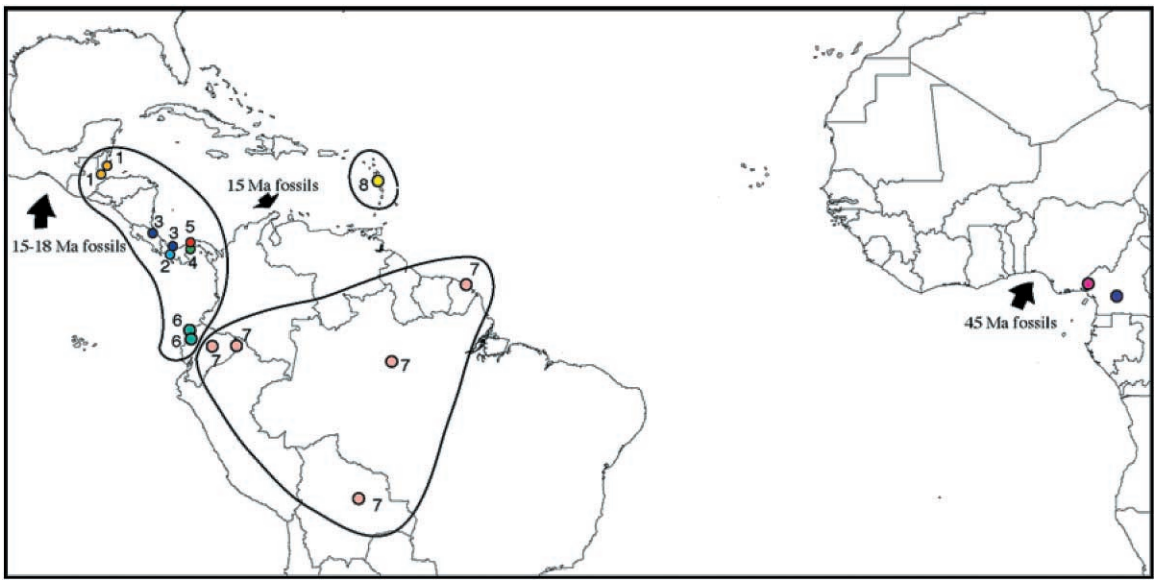

Figure 1: Leaf collections and fossil records of *Symphonia globulifera* in the Neotropics and Africa. The eight Neotropical ITS haplotypes are numbered as in table 1. The three Neotropical *S. globulifera* clades (cis-Andes, trans-Andes, and West Indies) are outlined (see fig. 2 for phylogenetic relationships). The arrows point to locations of the earliest regional fossils of *Symphonia* pollen for the three continents in which *S. globulifera* occurs.

Forest Science on Barro Colorado Island (BCI), Panama, and in Amazonian Ecuador (Yasuní). We made additional collections at distances of 10–50 km from the permanent inventory plots in west Ecuador, Brazil, and Panama to determine levels of regional ITS variation.

Laboratory Methods

DNA was extracted using the DNeasy kit (Qiagen, Valencia, Calif.). The internal transcribed spacers ITS1 and ITS2 and the 5.8S ribosomal gene were amplified using the ITS4 (White et al. 1990) and ITSi (Urbatsch et al. 2000) primers, which anneal to the flanking 18S and 26S ribosomal genes. Polymerase chain reactions were performed on an MJ Research thermal cycler with the following conditions: 94°C for 4 min, followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 3 min. The amplification products were separated from low melting point agarose using Gelase (Epicentre Technologies, Madison, Wis.) and sequenced using Big Dye chemistry (Applied Biosystems, Foster City, Calif.). The ITS sequences were edited and aligned with Sequencher 4.1 (Gene Codes, Ann Arbor, Mich.) and MacClade 4.0 (Maddison and Maddison 2000).

Phylogenetic Analyses

To analyze the phylogenetic relationships of the *S. globulifera* ITS lineages, we used ITS sequences from several Madagascan congeners and *Montrouziera sphaeroidea* Planch. ex. Planch. & Triana from New Caledonia (Gustafsson and Bittrich 2003) as outgroup taxa. Strong morphological evidence indicates that *Symphonia* is sister to one or more genera in the tribe Symphonieae, which includes *Montrouziera* (Gustafsson et al. 2002; Stevens, in press). The Madagascan *Symphonia* species used in our analysis, followed by herbarium accession in brackets, are as follows: *Symphonia verrucosa* (Hils. & Boj. ex Planch. & Triana) Vesque [T. C. Flores & J. Andriantiana 111], *Symphonia microphylla* Benth. & Hook. f. ex Vesque [T. C. Flores & J. Andriantiana 99], *Symphonia fasciculata* (Planch. & Triana) Vesque [K. Abdul-Salim et al. 144], and *Symphonia urophylla* (Decne. ex Planch. & Triana) Vesque [K. Abdul-Salim & R. Ranaivojaona s. n.]. All vouchers for these species are located at the Harvard University Herbaria.

We performed a Bayesian phylogenetic analysis using MrBayes 2.1 (Huelsenbeck and Ronquist 2001) following the analytical recommendations outlined in the MrBayes 2.1 manual. The Kimura three-parameter (K81) model of nucleotide substitution (Kimura 1981) provided the best fit for the DNA sequence data, as determined by a series of likelihood ratio tests implemented in Modeltest v. 3.06 (Posada and Krandall 1998). MrBayes 2.1 implements only the HKY85 (Hasegawa et al. 1985) and the general time reversible (GTR; Tavaré 1986) models, however, so we adopted GTR since it more appropriately approximates

Table 1: Genetic distance among the Neotropical *Symphonia globulifera* ITS haplotypes (represented by a haplotype number and a geographical region)

ITS haplotype	GenBank accession no.	1	2	3	4	5	6	7	8
1. Belize	AF479781		.01158	.00825	.02123	.01829	.00825	.02677	.04408
2. Chiriquí, PA	AF479782	8 (2)		.00329	.01660	.01492	.00659	.02165	.01997
3. Costa Rica	AF479783	6 (1)	4 (2)		.01325	.01158	.00329	.01828	.01661
4. Campana, PA	AJ312606	14 (4)	11 (4)	8 (2)		.00164	.01660	.03191	.03022
5. BCI, PA	AF479784	13 (4)	10 (4)	7 (2)	1 (0)		.01492	.03019	.02849
6. West Ecuador	AF479785	7 (3)	5 (3)	2 (1)	12 (5)	11 (5)		.02166	.01998
7. Cis-Andes	AF479786	17 (5)	13 (5)	11 (4)	20 (7)	19 (7)	14 (6)		.00824
8. Dominica	AF479787	16 (4)	12 (4)	10 (3)	18 (6)	17 (6)	12 (5)	5 (3)	
Symphonia urophylla	AF479788	28 (10)	26 (10)	24 (9)	29 (10)	28 (10)	27 (11)	18 (9)	17 (6)

Note: Genetic distance measured as absolute nucleotide differences (below diagonal), with transversions in parentheses, and as a Kimura three-parameter distance (above diagonal). The Madagascan congener *Symphonia urophylla* is provided in the bottom row. PA = Panama, BCI = Barro Colorado Island.

the K81 model. We made preliminary runs to determine the asymptote of the fluctuating likelihood values of the Bayesian trees. The asymptote was consistently observed before 400,000 cycles, so we ran the Markov chain analysis for 1×10^6 cycles and sampled one tree every 100 cycles once 400,000 cycles had passed. The 6,000 sampled trees were used to generate a 50% majority rule consensus tree in which posterior probabilities of each clade were indicated by the clade's representation (as a percentage) of Bayesian trees. These are true probabilities given the assumptions of the GTR model (Huelsenbeck and Ronquist 2001). Thus, probabilities of 95% or greater were considered significant. We also performed a maximum likelihood (ML) analysis in PAUP 4.04b8 (Swofford 1998) using the K81 model. We obtained bootstrap estimates of branch support based on 1,000 replicates of the ML tree. Gaps and ambiguous characters were excluded from the phylogenetic analyses.

Molecular Clock Tests and Divergence Time Estimates

We performed a likelihood ratio test of the hypothesis of equal substitution rates among *S. globulifera* lineages (Felsenstein 1988), which led to a rejection of the molecular clock hypothesis ($P < .01$). In order to utilize the temporal information in our data, we used the nonparametric rate smoothing (NPRS) technique of Sanderson (1997), which uses a criterion that maximizes the autocorrelation of substitution rates within a clade and thereby permits a molecular clock analysis of DNA sequence data that exhibit rate heterogeneity. We computed a mean divergence time estimate and 95% confidence intervals for each major node of the *S. globulifera* clade by performing the NPRS procedure on 100 bootstrapped trees generated by PAUP 4.04b8. As in Soltis et al. (2002), we transformed the ML trees into ultrametric trees using the NPRS method implemented in TreeEdit version 1.0 a10 (A. Rambaut and M. Charleston, Oxford University; available at http://evolve.zoo.ox.ac.uk/software). To provide alternative estimates of substitution rates and divergence times, we performed a penalized likelihood (PL) analysis (Sanderson 2002) of the same data set using the program r8s v. 1.60 (M. Sanderson, University of California, Davis; available at http://ginger.ucdavis.edu/r8s). The PL method combines a parametric model permitting a different substitution rate on every branch with a nonparametric roughness penalty, and it introduces a cost if rates change too quickly from branch to branch. The relative contribution of these two components to the model is determined by a smoothing parameter. We used the truncated newton optimization routine and a smoothing parameter of 744, which we obtained on the basis of the results of a cross-validation test (Sanderson 2002). For both the NPRS and PL approaches, we fixed the ancestral node of the cis- and trans-Andean lineages at 15 Ma, since *Symphonia* pollen was widespread in Mesoamerica and South America by this time.

In order to estimate divergence times for *S. globulifera* lineages independent of the *Symphonia* fossil record, we applied ITS substitution rates published for other angiosperm taxa that have been calibrated using either fossil or biogeographic data. Reference to the review of the plant ITS molecular clock published by Richardson et al. (2001) suggests that published rate estimates for ITS fall into two categories. The ITS clock for 12 taxa with generation times <3 yr averaged 4.08×10^{-9} substitutions per site per year (s/s/yr), whereas the substitution rate was an order of magnitude slower for trees in the Winteraceae with generation times in excess of 10 yr ($3.2–5.7 \times 10^{-10}$ s/s/yr). Suh et al. (2000) have published a rate estimate of 0.85×10^{-10} s/s/yr for maples (*Acer*), another tree taxon with a generation time that exceeds 10 yr. We have reanalyzed published ITS data for angiosperm taxa meeting the following criteria: GenBank accessions for ITS1, 5.8S, and ITS2 and

a published fossil or biogeographic record of the minimum divergence time separating the species for which ITS evolutionary rates have been calculated. Our analysis considers the combined ITS1, 5.8S, and ITS2 sequence for four groups trimmed of flanking ribosomal DNA sequence and excluding gaps (table B2 in the online edition of the *American Naturalist*). All ITS sequences were aligned visually using the program Se-Al v. 2.0 (A. Rambaut, Oxford University; http://evolve.zoo.ox.ac.uk/software.php). Rates of nucleotide substitution (D) were calculated using the formula $D = K/2T$, where T is the divergence time and K is the genetic distance estimated using the K81 model of nucleotide substitution.

The ITS divergences among maples (*Acer*) were calculated using subgenera that separated during the early Eocene (50 Ma) and are based on leaf fossils (Suh et al. 2000). The ITS divergence between *Pseudowintera* (Winteraceae) and its sister clade comprising the genera *Bubbia* and *Zygogynum* was based on Oligocene (30–35 Ma) macrofossils of *Pseudowintera* (Suh et al. 1993). The ITS divergence between Hawaiian silverswords (*Dubautia*) and their California tarweed progenitor *Madia* was based on the 15 Ma origin of the summer-dry floristic province of western North America to which the tarweeds are adapted (Baldwin and Sanderson 1998). The ITS divergence between birches (*Betula*) and alders (*Alnus*) is based on fossil pollen characteristic of both genera from the Late Cretaceous (~70 Ma; Savard et al. 1993).

Results

Sequence Variation

The *Symphonia globulifera* ITS region varied in size from 645 to 650 base pairs (bp) reflecting a few insertion/deletions: ITS1 (251–257 bp), the 5.8S ribosomal gene (164 bp), ITS2 (206–207 bp), and some flanking ribosomal gene sequence. The *S. globulifera* ITS sequences contained 41 variable sites, excluding indels, and 19 were phylogenetically informative. Several nucleotide sites in the *Symphonia* data set appeared as double peaks in the chromatograms. These ambiguous sites were excluded from analysis because we could not determine whether they represented sequencing artifacts or the rare heterozygote in the ribosomal gene family. We recovered eight geographically structured ITS haplotypes in Neotropical *S. globulifera* (fig. 1; table 1). No polymorphism was detected within sample sites despite examination of moderately large samples of trees from the permanent forest inventory plots on Barro Colorado Island, Panama ($N = 18$), and Yasuní, Ecuador ($N = 15$). Individuals were collected across moderate distances at some sample sites and also demonstrated no differences in their ITS sequences: Belize (20 km between samples), west Ecuador (20 km), central Panama (50 km), Brazil (60 km), and northwest Panama/Costa Rica (200 km). We observed autapomorphic indels defining three Neotropical *S. globulifera* ITS lineages: cis-Andean (5-bp deletion), coastal Ecuador (1-bp insertion), and Belize (1-bp insertion; fig. 2).

Phylogeny and Phylogeography

Our analyses provide strong statistical support for the monophyly of *S. globulifera*, using four of the Madagascan congeners or *Montrouziera sphaeroidea* to root the tree (fig. 2). The clade representing all *S. globulifera* ITS haplotypes received a posterior probability of 1.0 in the Bayesian analysis and ML bootstrap support of 100%. Furthermore, both New World and African *S. globulifera* shared a 1-bp deletion, which establishes an indel synapomorphy distinguishing this species from the 10 Madagascan congeners examined to date (Abdul-Salim 2002). The reciprocal monophyly of the African and Neotropical ITS lineages, however, is not supported at the $P < .05$ level. Nonetheless, 77% of the sampled Bayesian trees are reciprocally monophyletic. This result raises the important question of whether the lack of significance owes to the limited sample of nucleotides or to multiple trans-Atlantic dispersal events during the complex expansion history of *S. globulifera*.

The question of reciprocal monophyly of New and Old World *S. globulifera* aside, phylogeographic analysis of the Neotropical representatives of the species revealed three major ITS lineages: trans-Andes (Mesoamerica + west Ecuador), cis-Andes (Amazonia + French Guiana), and West Indies (Dominica; fig. 1). The average K81 genetic distance based on total nucleotide differences (table 1) in the 460 bp of ITS1 and ITS2 between the cis-Andean lineage and the trans-Andean ITS clade is 3.4%; the distance between the Dominican and cis-Andean lineages is 1.1%. The distinctiveness of the cis-Andean clade is further supported by a 5-bp deletion in ITS1 for the 27 trees collected across 2,500 km of lowland forest. The phylogenetic relationship among the three Neotropical ITS clades of *S. globulifera* is unresolved, reflecting either limited nucleotide sample size or a history of New World expansion that was fast relative to the ITS nucleotide substitution rate.

Only the trans-Andean clade contained multiple ITS haplotypes. The monophyly of the trans-Andean clade was supported by a posterior probability of 1.0 in the Bayesian analysis and ML bootstrap support of 100%. Except for the central Panama samples (Campana, Ft. Sherman, Barro Colorado Island), phylogenetic relationships among haplotypes within the trans-Andean ITS clade were not well resolved. The average genetic distance among trans-Andean ITS haplotypes was 1.7%, with a range from 0.2%

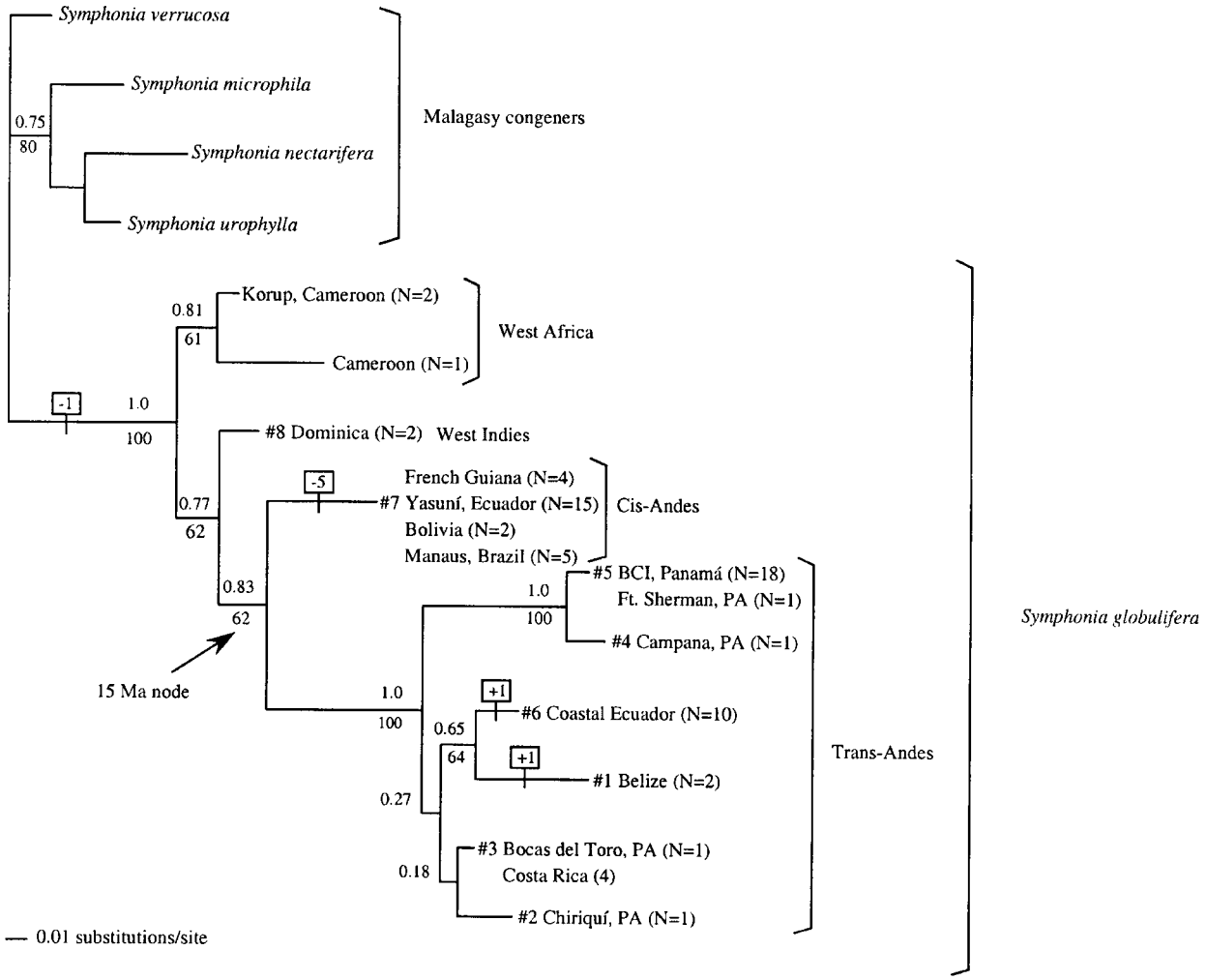

Figure 2: Bayesian phylogenetic tree of representative *Symphonia* species from Madagascar and *Symphonia globulifera* populations from Africa and the New World on the basis of ~645 base pairs of nuclear ribosomal and spacer (ITS) DNA sequence. The same topology of the intraspecific *S. globulifera* clade was obtained by rooting the tree with *Montrouziera sphaeroidea*. The Bayesian posterior probabilities of each node are indicated above the branch preceding the node. The ML bootstrap values based on 1,000 replicates are provided below the Bayesian probabilities when >50. The haplotype number is positioned at the tip of each Neotropical branch and corresponds to the map in figure 1. The rectangular boxes along some branches represent insertions (+bp) or deletions (−bp) relative to the consensus sequence. Sample sizes are indicated in parentheses after each collection locale. The arrow points to the node between the cis- and tran-Andean clades, which was set at 15 Ma to estimate absolute rates of ITS nucleotide substitution.

(1-bp difference) between haplotypes 4 (Campana, Panama) and 5 (Barro Colorado Island, Panama) to 3% (14 bp) between haplotypes 4 (Campana, Panama) and 1 (Belize; table 1). Additionally, a 1-bp insertion distinguished the 10 individuals collected in coastal Ecuador, and a different 1-bp insertion was observed in the two Belize trees.

ITS Molecular Clocks

Our reanalysis of angiosperm ITS divergence utilizing the K81 nucleotide substitution model and combined ITS1, 5.8S, and ITS2 data yielded two distinct evolutionary rates that could be applied to the *Symphonia* data. The evolutionary rate for herbaceous plants represented by the Hawaiian silverswords is 2.64×10^{-9} s/s/yr. The mean rate for the arboreal plants we examined is 6.39×10^{-10} s/s/yr and was based on rates of 6.03×10^{-10} for the Winteraceae, 6.5×10^{-10} for *Acer*, and 6.64×10^{-10} for the Betulaceae. The PL method (Sanderson 2002) applied to the *S. globulifera* clade provided a mean rate of ITS nucleotide substitution of 7.04 ($\pm$0.24) $\times 10^{-10}$ s/s/yr. The PL rate estimate, while accounting for the rate heterogeneity in

the *Symphonia* ITS data set, falls between the fast (herbaceous) and slow (long-lived tree) published ITS rate estimates.

Divergence Times of Neotropical Symphonia globulifera *Lineages*

The average genetic distance between the cis- and trans-Andean *S. globulifera* ITS sequences was 0.023 substitutions per site under the K81 model of nucleotide substitution. With the ancestral node of the cis- and trans-Andean lineages fixed at 15 Ma, the NPRS analysis placed the divergence of *S. globulifera* (African and New World representatives) from the Madagascan congener *Symphonia urophylla* at 28.52 (±3.35) Ma, postdating the mid-Eocene (~45 Ma) records of *Pachydermites diederexii* from Nigeria (Germeraad et al. 1968; Jan-du-Chene et al. 1978; table 2). The estimated divergence time of the Neotropical and African *S. globulifera* was 17.36 (±1.53) Ma and is followed by the divergence of the West Indian (Dominica) *S. globulifera* from the continental New World lineages at 16.27 (±1.32) Ma. The NPRS analysis placed the expansion and divergence of the trans-Andean clade at 7.18 (±1.26) Ma, antedating the numerous fossil records of *P. diederexi* from Pliocene sediments from Mexico and Central America (Graham 1976; Graham and Dilcher 1998) but postdating the earliest Mesoamerican records of *P. diederexii* (15.5–18.2 Ma) from Mexico. The NPRS estimates of divergence times fall between estimates derived from the arboreal and herbaceous ITS clocks for all dated nodes and are similar to the estimates obtained using the PL method (table 2).

Discussion

The internal transcribed spacer (ITS) region has helped resolve phylogenetic relationships within hundreds of seed plant families and genera (Baldwin et al. 1995). Our study demonstrates that the ITS region is also useful for resolving historical relationships among populations of relatively old and widespread species. This was a novel finding, since many studies have found a lack of ITS variation among some congeneric species (e.g., within *Acer* [Suh et al. 2000], *Saxifraga* [Vargas et al. 1999], *Dendroseris* [Sang et al. 1994], *Dubautia* [Baldwin and Sanderson 1998], *Inga* [Richardson et al. 2001]) or even among genera in the Winteraceae (Suh et al. 1993). The patterns of *Symphonia globulifera* differentiation revealed by the ITS sequences indicate that range expansion of this species has been facilitated by marine dispersal, while regional differentiation appears tied to geographic heterogeneity associated with tropical mountains. While the phylogenetic details of *S. globulifera*'s early dispersal history are obscure, our ITS results coupled with the fossil record provide clear and compelling evidence of a Tertiary expansion of *S. globulifera* in the Neotropics leading to three genetically divergent and geographically structured evolutionary lineages. The prevailing taxonomy of this widespread and morphologically uniform species implies a history of recent dispersal or contemporary gene flow that is falsified by our molecular systematic analysis. In turn, our *S. globulifera* results raise significant questions about the degree of evolutionary connectedness among other widespread conspecific populations inhabiting Neotropical lowland rain forests.

The Early Expansion History of Symphonia globulifera

The relatively low level of ITS divergence between African and New World *S. globulifera* discounts a Gondwana vicariance origin (>90 Ma) for the species' trans-Atlantic disjunction (table 2). Strong inference from the genetic and fossil evidence indicates that marine dispersal has permitted *S. globulifera* to migrate between Africa and the New World as well as expand its distribution within the Neotropics. The remarkable trans-Atlantic disjunction of *S. globulifera* has garnered the attention of numerous authors (Germeraad et al. 1968; Raven and Axelrod 1974;

Table 2: Comparison of divergence time estimates (Ma) for the major ITS clades of *Symphonia globulifera*

Common ancestor node	NPRS method	PL method 7.04 (±.24) × 10^{10} s/s/yr	Silversword ITS clock 2.64 × 10^{-9} s/s/yr	Arboreal ITS clock 6.39 × 10^{-10} s/s/yr
Symphonia urophylla/ Symphonia globulifera	28.52 (±3.35)	32.37	12.3	50.7
Africa/Neotropics	17.36 (±1.53)	17.79	10.1	43.0
Dominica/Trans-Andes	16.27 (±1.32)	16.21	8.9	36.6
Cis-Andes/Trans-Andes	15.00 (fixed)	15.00 (fixed)	8.1	36.0
Trans-Andes clade	7.18 (±1.26)	5.03	3.9	16.1

Note: Estimates based on the NPRS method of Sanderson (1997) and the PL method of Sanderson (2002) were obtained by fixing the age of the common ancestor of the cis- and trans-Andean ITS lineages at 15 Ma (see "Material and Methods"). The divergence estimates in columns 3 and 4 are based on the application of an ITS molecular clock derived for herbaceous angiosperms represented by the Silversword alliance (column 3) and long-lived woody angiosperms (column 4) to the mean divergence of *S. globulifera* clades using Kimura three-parameter distances.

Gentry 1993; Morley 2000). In the words of Germeraad et al. (1968, p. 278): "The means by which this dispersal took place are as yet wholly obscure. At this late stage in the Tertiary the topography may have been similar to present-day conditions, and the chances of (*Symphonia*) seeds crossing the Atlantic must have been extremely small. That this nevertheless occurred can hardly be doubted, however, and the long time delay is a silent witness of the numerous unsuccessful attempts which must have preceded the final success." Given the lack of avian migratory routes across the Atlantic and gut passage rates for seeds that range from minutes to hours (e.g., Holbrook and Smith 2000), transoceanic expansion mediated by birds seems unlikely. Oceanic seed dispersal is also unlikely because the *Symphonia* seed does not have dormancy and dies quickly with desiccation (Maury-Lechon et al. 1980). We consider whole trunks or roots rather than seeds as more plausible vectors of marine dispersal, a hypothesis supported by the observation that *S. globulifera* thrives along rivers and can propagate vegetatively (Scarano et al. 1997).

Although the Bayesian analysis provided strong statistical support for the monophyly of all *S. globulifera* ITS haplotypes when rooted with the Madagascan *Symphonia* congeners and *Montrouziera*, the monophyly of the Neotropical ITS haplotypes was not supported at the $P < .05$ level. Reciprocal monophyly of the African and Neotropical ITS lineages would be expected in the case of a single trans-Atlantic dispersal or relatively ancient cessation of gene flow. The polytomy of the African and three Neotropical ITS clades, however, suggests that *S. globulifera* achieved much of its contemporary distribution early in its expansion history but quickly relative to the rate of ITS nucleotide substitutions. More data are needed to determine whether *S. globulifera* has dispersed across the Atlantic one or more times.

Our results demonstrate that marine dispersal has played an important and unanticipated role in the continental expansion of *S. globulifera* in the Neotropics. In the first instance, phylogenetic analysis and molecular clock dating of the three principal New World *S. globulifera* clades indicate that they last shared a common ancestor at a time when Mesoamerica and South America were separated by a deep oceanic channel (>3.1 Ma), a condition persisting for most of the Tertiary (Coates and Obando 1996). This inference is further supported by the first occurrence of *Pachydermites diederexi* in South America and Mexico in the mid-Miocene, more than 10 Ma before the formation of the Panama land bridge. The occurrence of *S. globulifera* on Dominica, an oceanic island of Oligocene volcanic origin (Iturraldi-Vinent and MacPhee 1999), represents a probable third instance of marine colonization. Although the spread of *S. globulifera* into the West Indies during the slave trade cannot be entirely discounted, marine dispersal seems to be more parsimonious given that the species has no documented history of cultivation by humans, and the Dominica ITS haplotype is not closely related to two African ITS haplotypes sequenced to date.

Phylogeography

The most striking result of our phylogeographic analysis of *S. globulifera* is the contrast in the spatial distribution and ITS haplotype diversity of cis- and trans-Andean populations. Our samples of trans-Andean *S. globulifera* displayed moderate levels of phylogeographic separation among ITS haplotypes distributed across ~2,000 km of coastal forest from Belize to Ecuador, which stands in stark relief to the complete absence of geographic structure and ITS haplotype diversity across 2,500 km of lowland forest east of the Andes. The phylogeographic complexity of the trans-Andean clade suggests that expansion of *Symphonia* in this region considerably predated the contemporary expansion of the species across Amazonia and/or that the greater geographical heterogeneity of Mesoamerica compared with the Amazon basin has caused a reduction in gene flow leading to increased population subdivision. Similar patterns of population genetic structure in Mesoamerica have been shown in other taxa, including trees (Aide and Rivera 1998; Gillies et al. 1999; Cavers et al. 2003; Novick et al. 2003), fish (Bermingham and Martin 1998; Perdices et al. 2002), howler monkeys (Cortés-Ortiz et al. 2003), and snakes (Zamudio and Greene 1997). There are no published phylogeographic studies of which we are aware that represent the breadth of the Amazon basin. However, a recent mtDNA-based phylogeographic analysis of *Amazona ochrocephala* parrots presents a similar pattern of strong phylogeographic structure in Mesoamerica contrasting the absence of geographic structure across 2,000 km of Amazonia, in this case covering the southern half of the Amazon basin from the mouth of the Amazon to Bolivia and Peru (Eberhard and Bermingham, in press). Moreover, a recent microsatellite-based analysis of mahogany (*Swietenia macrophylla*) distributed along the southern edge of the Amazon basin also failed to demonstrate strong geographic subdivision in this region (Lemes et al. 2003), whereas analysis of Mesoamerican mahogany based on the same markers identified four regional population groups (Novick et al. 2003).

The most prominent physical barriers to the dispersal of Mesoamerican rain forest trees are mountains and xeric habitats, which tend to covary in distribution because of the influence of mountains on regional patterns of precipitation. Mountains are particularly effective as biotic barriers in the tropics because the altitudinal temperature

gradient imposes a more severe physiological constraint on the movement of lowland tropical organisms in comparison to lowland temperate organisms adapted to the temperature swings of the seasons (Janzen 1967). For example, the Andean cordillera appears to form a particularly strong barrier to the movement of *Symphonia*; cis- and trans-Andean populations of *S. globulifera* apparently have not exchanged ITS genes since their establishment on the east and west flanks of the mountains. The strength of the Andean barrier probably owes to the lack of mountain passes of sufficiently low elevation to connect the lowland rain forests on either slope. The lower and younger Talamanca range extending from Costa Rica to western Panama separates ITS haplotype 2 in the Chiriquí highlands on Panama's Pacific slope from haplotype 3 collected in the Caribbean lowlands of western Panama and Costa Rica. More complete sampling of *S. globulifera* across its trans-Andean distribution would be required to complete our understanding of the species phylogeographic history, but it is worth noting the rough correspondence of ITS haplotype distribution to the biogeographic zones proposed as Mesoamerican glacial forest "refugia" by Whitmore and Prance (1987). On the basis of a compilation of paleoclimatic, geological, and soil data, these authors argued that moist tropical forest persisted in three regions during periods of glacial cooling: one stretched from southern Mexico to northern Honduras (the area harboring Belize haplotype 1), a second from southern Nicaragua to western Panama (haplotypes 2 and 3), and the third in the Chocó region of eastern Panama to the Pacific slope of Ecuador (haplotype 6).

In addition to the putative role of mountains and climate in promoting the phylogeographic structure of *S. globulifera*, we suggest that the especially high lineage diversity within Panama may reflect the complex geological history of the land bridge. The hills presently cut by the Panama canal are remnants of an archipelago that supported large North American herbivores such as horses and rhinoceroses in the mid-Miocene (~18 Ma; Whitmore and Stewart 1965). If *S. globulifera* inhabited these islands, populations could have differentiated before the rise of the intercontinental land bridge at 3.1–3.5 Ma, at which time they would also have come into contact with Mesoamerican and South American floral elements (Gentry 1982). The complete admixture of forests and haplotypes may take a long time, especially if the habitats required for the invading species or populations are already saturated (MacArthur 1972). In this connection, it is worth noting that the continuous forest along the Atlantic verdant of Panama harbors *S. globulifera* ITS haplotypes distinguished by seven substitutions between the Bocas del Toro population in western Panama (haplotype 3 in fig. 1) and the Fort Sherman and BCI populations of central Panama (haplotype 5).

The complete absence of phylogeographic structure over the vast distances separating the cis-Andean populations was unexpected given the long fossil history of *S. globulifera* in the region. *Symphonia globulifera* populations are not contiguous across this area: dry habitats intersect the distribution of rain forests from French Guiana to the Amazon basin. Nevertheless, habitat differences in the Amazon basin and Guiana shield probably represent minor biogeographic barriers compared with the mountain ranges that subdivide Mesoamerican rain forests. Furthermore, the historical dynamics of the Amazon River may have promoted the population expansion of *S. globulifera* populations. For example, the paleo-Amazon River flowed west and then north before assuming its contemporary eastern drainage pattern between the Guiana and Brazilian shields roughly 8 Ma (Lundberg et al. 1998). Historically, the drainage patterns of South America's large rivers have changed, and thus they may have permitted the quick spread of riparian populations of *S. globulifera* across vast watersheds. The lack of ITS nucleotide polymorphism in the cis-Andean ITS lineages may be the signature of a relatively recent population expansion of *S. globulifera* in Amazonia or extensive contemporary gene flow.

Geological Age of Tropical Tree Diversity

The ITS phylogeography of *S. globulifera* reflects the imprint of plate tectonics (producing the Panamanian land bridge), mountain ranges, and marine dispersal on the population structure of Neotropical rain forest trees. The morphological stasis of *S. globulifera* over this period stands in contrast to the Neotropical tree genus *Inga*, which has radiated into roughly 300 species since the mid-Miocene and in many cases exhibits few or no ITS nucleotide substitutions between sister species (Richardson et al. 2001). While the rapid morphological radiation of *Inga* may characterize many species-rich Neotropical lineages, we suggest that the great age and morphological stasis of *S. globulifera* may characterize species-poor lineages with widespread distributions. More generally, the richness of tree genera in tropical rain forest communities indicates that most Neotropical plant diversity is of Tertiary origin (Ricklefs and Schluter 1993) and holds open the possibility that many widespread species may also be old. The 25-ha inventory plot in Yasuní, Ecuador, for example, contains more than 1,100 tree species in 369 genera, of which 161 are represented by a single species, one of which is *S. globulifera* (Bermingham and Dick 2001; Center for Tropical Forest Science, unpublished data). The geographic distribution of these genera provides further evidence of their

antiquity: at least 105 of the 369 tree genera in the Yasuní plot also occur in the Old World tropics (C. W. Dick, unpublished data).

It is important to understand the age structure of Neotropical plants because it bears on the perennial question of why there are so many kinds of tropical trees. Deep ages provide opportunities for species to expand their distributions through rare dispersal events and the invasion of new habitats. The combination of age and increased spatial range provides time and opportunity for allopatric divergence, ecological diversification, and increased genetic or morphological diversity and divergence. In turn, these varied factors set the stage for reproductive isolation, sympatric overlap, and net increases in local biodiversity. In the case of *S. globulifera*, the age and divergence of its regional populations are amply demonstrated by the molecular and fossil record, but we have yet to witness the sympatric distribution of divergent phylogenetic lineages that would herald a net increase in α diversity. Nonetheless, the Neotropical history of *S. globulifera* provides a fresh perspective on patterns of β diversity among lowland Neotropical sites. The contrast in ITS diversity between cis- and trans-Andean *Symphonia* is paralleled by a reduction in the β diversity of tropical trees in Amazonian plots separated by 1,400 km in comparison to sites in Panama separated by only 50 km (Condit et al. 2002). Greater environmental heterogeneity in Panama was posited as the probable cause of its high β diversity, but our study of *S. globulifera* suggests that biogeographic history, perhaps in conjunction with environmental heterogeneity, might also explain the pattern. We suggest that high β diversity of Panamanian forests stems from the geographical role of Panama as a mixing ground for independently derived floras. This hypothesis implies a very long approach to equilibrium for tree populations, their haplotype diversity, and the ecological communities they comprise.

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