Repeated Evolution of Dioecy from Monoecy in Siparunaceae (Laurales)

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Abstract.—Siparunaceae comprise Glossocalyx with one species in West Africa and Siparuna with 65 species in the neotropics; all have unisexual flowers, and 15 species are monoecious, 50 dioecious. Parsimony and maximum likelihood analyses of combined nuclear ribosomal ITS and chloroplast trnL-trnF intergenic spacer sequences yielded almost identical topologies, which were used to trace the evolution of the two sexual systems. The African species, which is dioecious, was sister to all neotropical species, and the monoecious species formed a grade basal to a large dioecious Andean clade. Dioecy evolved a second time within the monoecious grade. Geographical mapping of 6,496 herbarium collections from all species sorted by sexual system showed that monoecy is confined to low-lying areas (altitude < 700 m) in the Amazon basin and southern Central America. The only morphological trait with a strong phylogenetic signal is leaf margin shape (entire or toothed), although this character also correlates with altitude, probably reflecting selection on leaf shapes by temperature and rainfall regimes. The data do not reject the molecular clock, and branch lengths suggest that the shift to dioecy in the lowlands occurred many million years after the shift to dioecy in the ancestor of the Andean clade. [Altitudinal distribution of sexual systems; dioecy; molecular clock; monoecy; sexual system evolution.]

About 6% (14,620 of 240,000) of the species of flowering plants are dioecious, and about 7% (959 of 13,500) of the genera are completely or partly dioecious (Renner and Ricklefs, 1995). Comparative evidence suggests that dioecy has evolved many times from perfect-flowered (monoclinous) or monoecious (with diclinous flowers on each plant) ancestors (Lewis, 1942; Charlesworth and Charlesworth, 1978; Lloyd, 1980). Here we study a small tropical family with diclinous flowers and monoecious or dioecious species, using a phylogeny to infer the frequency of shifts between these sexual systems. We also address the possible relation between an ecological factor (altitude) and switches in sexual system and discuss differences between some major evolutionary pathways to dioecy. Two key terms, paradioecy and gynodioecy, need to be defined at the outset. Paradioecy is the inconstant presence of male or female flowers in the males and females of dioecious species, with the inconstancies being of similar magnitude in both sexes (Lloyd, 1972a). Gynodioecy is the regular coexistence of perfect-flowered and pistillate individuals (Darwin, 1877; Percival, 1965; Lloyd, 1976; Delannay, 1978; Mayer and Charlesworth, 1991; Grant, 1999). Some times, the term is used more broadly to apply to populations composed of female and hermaphroditic individuals, irrespective of whether the genetic hermaphrodites have bisexual or unisexual flowers (Lloyd, 1980; Barrett, 1998; Sakai and Weller, 1999; Sarkissian et al., 2001). This broadening of the meaning of gynodioecy affects the debate about two of the evolutionary pathways to dioecy—the gynodioecy/dioecy pathway and the monoecy/paradioecy/dioecy pathway—a point we take up in the discussion.

Our study system is Siparuna, a genus with 50 dioecious and 15 monoecious species that do not vary in pollination, seed dispersal, or woodiness. All species are gall midge–pollinated, animal-dispersed shrubs or trees of humid forests (Feil and Renner, 1991; Feil, 1992; Renner et al., 1997; Schulz-Burck, 1997). That monoecy or dioecy (or both) might have evolved repeatedly within the genus is suggested by the pattern of leaf margin variation (photos of most leaves appear in Renner and Hausner [1997] at http: www.siparuna.com). All monoecious species have entire leaf margins, whereas all but two dioecious species have toothed margins. The two dioecious species with entire margins could have originated from entire-margined ancestors, representing a transition from monoecy to dioecy separate from the one in the toothed clade. Leaf margin shape, however, is highly correlated with temperature and rainfall (Wolfe, 1990; Wiemann et al., 1998), the proportion of entire-margined leaves in a flora being the best single character for estimating mean annual temperature. Independent evidence is therefore needed to test the hypotheses that, in Siparuna, leaf margin shape carries phylogenetic signal and that its message (two sexual system switches) is trustworthy. Finally, we were interested in estimating the relative and absolute times of any switches in sexual system because simultaneous changes at particular times might argue for similar factors having selected for them.

Siparuna is distributed in tropical South America and is one of two genera of Siparunaceae, a family of Laurales (Renner, 1999). The other genus is Glossocalyx with a single species in lowland tropical West Africa that is dioecious and has toothed leaves (Fouilloy, 1974; an additional species is recognized by Fouilloy but is considered a synonym by Renner and Hausner, unpubl. manuscript). A third genus, Bracteanthus , is here found nested within Siparuna. The ranges and gross morphology of the species studied are known from ongoing monographic work (Renner and Hausner, 1997, 2000). Sexual systems are known from fieldwork on 13 dioecious species in Ecuador (Feil, 1992) , 6 monoecious species in Amazonian Brazil (Schulz-Burck, 1997), and herbarium study of all species. More than 8,000 herbarium collections of Siparunaceae have been studied for a monograph of the family. Flowers of Siparunaceae are a few millimeters in diameter and strictly unisexual. They are borne in cymes that in monoecious species usually bear a mixture of male and female flowers, either on the same peduncle or on a nearby peduncle. Because of the small size of the reproductive structures, herbarium sheets of Siparuna often bear several inflorescences and many flowers, thus allowing the dissection and gender assessment of flowers. Species represented by sufficient herbarium specimens can therefore be identified as either monoecious or dioecious.

Siparunaceae are isolated among Laurales in the morphology and strict unisexuality of their flowers, and molecular phylogenies show that their closest relative is a clade with bisexual flowers, the monotypic Chilean Gomortegaceae and the trans-Antarctic Atherospermataceae (Renner, 1999; Renner et al., 2000). Morphological studies within Siparunaceae have provided little insight into the direction of evolutionary change in sexual system or any other character. The last monograph of Siparuna (Perkins, 1901), perhaps surprisingly, used sexual system as the first criterion in the arrangement of species, followed by indumentum and androecial characters. Even when more characters are used, potentially monophyletic groups and their relationships are difficult to discern. Therefore, we used nuclear and chloroplast DNA sequences to construct a phylogenetic hypothesis that provides a framework in which to discuss the evolution of monoecy and dioecy in Siparunaceae.

MATERIALS AND METHODS

Taxon Sampling

Representatives of Siparunaceae and their outgroups were selected on the basis of earlier morphological and molecular analyses (Perkins, 1901; Renner and Hausner, 1997; Renner et al., 1997). Trees were rooted with Gomortega nitida based on the finding that monotypic Gomortegaceae and Atherospermataceae together are the sister group of Siparunaceae (we did not include Atherospermataceae sequences because their highly divergent internal transcribed spacer (ITS) sequences caused dubious alignments). The 21 ingroup taxa (Table 1) consist of 7 of the 15 monoecious species and 14 of the 50 dioecious ones. One additional dioecious species, S. brasiliensis, was sampled only for trnL-trnF, which placed it inside the Andean dioecious clade. Material of Siparunaceae is difficult to obtain because many of the 65 species are restricted to particular mountain ranges in Colombia, Ecuador, and Peru, where individuals often grow widely dispersed. Although our sample is thus biased towards widespread species, it does include the rarely collected Bracteanthus glycycarpus (D Siparuna glycycarpa) and S. sp. nov. aff. monogyna. Herbarium specimens of Siparuna did not yield usable DNA.

DNA Isolation, Amplification, and Sequencing

Total DNA was isolated from silica gel–dried leaves from single individuals by using DNeasy plant mini kits (QIAGEN, Valencia, CA) according to the manufacturer ’s instructions. DNA amplification by the polymerase chain reaction (PCR) followed standard protocols. The chloroplast trnL-trnF

TABLE 1. Species sequenced for this study with their voucher specimens, geographical and altitudinal ranges, and GenBank accession numbers. Vouchers for silica-dried leaves are in the herbaria listed in parentheses after each collection; for herbarium acronyms see the Index Herbariorum at http://www.nybg.org/bsci/ih/ih.html.

Species	Mating system[a]	Voucher specimen	Range and altitude	trnL-trnF spacer	ITS
Bracteanthus glycycarpus Ducke D Siparuna glycycarpa (Ducke) Renner and Hausner, ined.	M	Sothers and Silva 708 (INPA, MO)	Eastern Amazon basin; 60–500 m	AF289849	AF289828
Glossocalyx longicuspis Benth.	D	Bos 4659 (MO)	West Africa, lowlands	AF012405	AF289845
Gomortega nitida R. & P.	B	Rodriguez 3070 (CONC)	Chile, lowlands	AF012404	AF289846
Siparuna aspera (R. & P.) A. DC.	D	Madriñán, Renner and Cortes 1502 (COL)	Andes from Colombia to Peru; 350–3,000 m	AF040695	AF289834
Siparuna bifida (P. & E.) A. DC.	D	Pereira-Moura 1711 (R)	Amazon basin; 85–600(–1,100) m	AF289853	AF289832
Siparuna brasiliensis (Spreng.) A. DC.	D	Pignal 309 (MO, P)	Southeastern Brazil; 100–1,600 m	AF012408	—
Siparuna cervicornis Perkins	D	Renner and Hausner 1 (MO, QCNE)	Western Amazon basin; 100–550 m	AF289854	AF289833
Siparuna conica Renner and Hausner	D	Galdames 4325 (PMA)	Andes from Panama to Ecuador; 0–1,860 m	AF289855	AF289835
Siparuna cuspidata (Tul.) A. DC.	M	Costa and Assunção 373 (INPA, K, MO)	Amazon basin and South Central America; 9–700 m	AF289848	AF289826
Siparuna cymosa A. Tolm.	M	Assunção 374 (INPA, MO)	Eastern Amazon basin; 50–500 m	AF012407	AF289827
Siparuna decipiens (Tul.) A. DC.	M	Sothers 911 (INPA, MO)	Amazon basin; 100–700 m	AF289847	AF289825
Siparuna echinata (H.B.K.) A. DC.	D	Potthast 243 (MO, QCA)	Andes from Colombia to Ecuador; 1,250–3,800 m	AF040696	AF289836
Siparuna guianensis Aublet	M	Chanderbai 247 (MO)	Costa Rica to Bolivia; 0–1,600 m	AF289850	AF289829
Siparuna laurifolia (H.B.K.) A. DC.	D	Devia and Prado 2278 (MO, TULV, US)	Andes from Colombia to Ecuador; 300–2,900 m	AF289856	AF289837
Siparuna lepidota (H.B.K.) A. DC.	D	Ståhl 2245 (MO, QCA)	Andes from Colombia to Ecuador; 500–3,000 m	AF289857	AF289838
Siparuna aff. monogyna Jangoux	M	Vicentini 1010 (MO)	Amazon basin; 100–200 m	AF289851	AF289830
Siparuna muricata (R. & P.) A. DC.	D	Merello et al. 1102 (MO)	Andes in Ecuador, Peru, and Bolivia; 1,500–3,400 m	AF289858	AF289839
Siparuna pauciflora (Beurl.) A. DC.	D	Lohmann s.n. (MO frag.)	Central America; 0–1,200 m	AF289859	AF289840
Siparuna radiata (P. & E.) A. DC.	D	Renner and Hausner 5 (MO, QCNE)	Western Amazon basin; 50–1,500 m	AF289860	AF289841
Siparuna reginae (Tuo.) A. DC.	M	Sothers and Pereira 642 (INPA, K, MO, NY)	Amazon basin; 80–1,200 m	AF289852	AF289831
Siparuna sessiliflora (H.B.K.) A. DC.	D	Madriñán, Renner, Cortes 1504 (COL)	Andes in Colombia; 50–1,700 m	AF289861	AF289842
Siparuna stellulata Perkins	D	Croat and Whitehill 82743 (MO)	Andes from Venezuela to Ecuador; (400–) 1,200–2,600 m	AF289862	AF289843
Siparuna thecaphora (P. & E.) A. DC.	D	Lohmann s.n. (MO frag.)	Central America to Bolivia; 0–2,400 m	AF289863	AF289844

[a] B D flowers bisexual; D D dioecious; M D Monoecious.

intergenic spacer region was amplified with primers c and d of Taberlet et al. (1991). Because of the proximity of the binding site of primer c to the start of the trnL-trnF spacer, the 5^{0} end of the spacer could not always be sequenced. For the analyses, we therefore truncated the 5^{0} end of all species by "-27 base pairs (bp). The ITS regions ITS-1 and ITS-2 and the intervening 5.8S rDNA sequence of the 18S–26S nuclear DNA region were amplified with primers ITS-A and ITS-B (Blattner, 1999). PCR products were purified with QIAquick PCR purification kits or with QIAquick gel extraction kits (both from QIAGEN). Cycle sequencing of the amplified double-stranded products was conducted with the ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Perkin-Elmer, Foster City, CA), using the PCR primers and the internal primers of White et al. (1990) for ITS. The dye was removed by ethanol precipitation and samples were then run on an ABI 377 automated sequencer at the University of Missouri-St. Louis. Both strands were sequenced, and consensus sequences were constructed using Seqman II (DNAstar Inc., Madison, WI).

Phylogenetic Analyses

Sequences were aligned with ClustalW (version 1.75; Thompson et al., 1994) and adjusted manually. The data matrix consisted of 22 ITS-1, ITS-2, and 5.8S sequences, and 15 complete and 8 partial trnL-trnF sequences. Phylogenetic analysis of aligned sequences was conducted by using PAUP* version 4.0b8 (Swofford, 2001). Heuristic searches were run with 1,000 random taxon-addition replicates, holding 100 trees at each step, and using tree bisection-reconnection (TBR) swapping. The MulTrees, Collapse, and Steepest Descent options of PAUP were in effect during all searches, and gapped and multiple-state characters (ambiguous base calls) were treated as missing data. Characters were equally weighted and unordered, and state changes were interpreted under ACCTRAN optimization. Nonparametric bootstrap support was estimated based on 1,000 replications, closest taxon addition, and TBR swapping. Decay indices were obtained by running AutoDecay 4.0 (Eriksson, 1998) in PAUP.

Maximum likelihood (ML) analyses were performed on the combined matrix (after exclusion of gapped characters) with use of the general time-reversible model (GTR; Yang, 1994) , which was found to fit the data significantly better than less parameter-rich models (data not shown). The model included a discrete approximation of a gamma (0) distribution with four rates for modeling rate heterogeneity among sites and assumed that some fraction of sites remained invariant (P_{inv}). The starting tree for the ML analysis was a minimum-evolution tree determined by using log-determinant genetic distances (Lockhart et al., 1994), and the swapping strategy used was TBR swapping. All model parameters were estimated simultaneously with tree searching, using the empirically observed base frequencies. Because the interdependence of model parameters and tree topology is weak as long as strongly supported groups are maintained, parameters can be estimated without complete search (Yang and Kumar, 1996; Sullivanet al., 1999; Sanderson and Kim, 2000). We interrupted parameter estimation after 7,202 TBR swaps on the single tree in memory at the time. The estimated parameters were then used in ML searches with TBR swapping, using one of the six most-parsimonious trees as starting tree; searches were aborted when the likelihood scores had stopped improving for many hours.

Molecular Clock Analyses

Because Gomortega ITS differed considerably from Siparunaceae ITS, Gomortega was excluded from molecular clock analyses. To assess the severity of rate heterogeneity among the remaining sequences, we used a likelihood ratio test that compared the likelihoods of the single best trees obtained under clock versus nonclock models. If mutations accumulate randomly, these likelihoods will not differ significantly. To conduct this analysis, we again excluded gapped characters and ran a heuristic search with the same model parameters as before, except that a clock was enforced. TBR swapping on the clock tree was complete after 11,846 rearrangements. The likelihood ratio test statistic is calculated as $-2 (\ln L_0 - \ln L_1)$, where L_0 and L_1 are the likelihoods under the null (clock) and alternative (nonclock) hypotheses. The significance of this value is judged by comparing it to a Â^{2}distribution with n degrees of freedom, n being the difference in the number of free parameters between the null and the alternative model. In our case, n is 21, because branch lengths are estimated for 43 branches in the unconstrained analysis, whereas the lengths of 22 branches are estimated under a clock constraint. We used binomial probability theory to estimate the standard deviation (SD) of the distance from a fixed calibration node to the tips and then used this value to obtain the SDs of the estimated ages. The binomial distribution describes the number of successes for independent trials. The GTR model of nucleotide divergence used takes multiple substitutions into account and thus estimates the number of independent nucleotide changes for which a binomial distribution is therefore appropriate. The number of invariant sites affects the probability of substitution at variable sites, but has little effect on the SD of the number of substitutions. According to the binomial distribution, the number of nucleotide substitutions (S) is equal to the product of the total number of nucleotides in a sequence (N) times the proportion of nucleotides substituted (p). Thus, $S = Np$. The SD of this value is the square root of $Np(1-p)$, or $SD(S) = \sqrt{Np(1-p)}$. The SD of the number of nucleotides substituted divided by the total number of nucleotides is the SD of the proportion of nucleotide substitutions. Thus, $SD(p) = \sqrt{p(1-p)/N}$.

Geographical Mapping of Leaf Margin Types and Sexual Systems

Geographical coordinates were available for 6,496 collections of Siparuna from a database built in connection with a monograph of the family (Renner and Hausner, unpubl. manuscript). These coordinates were processed with the ArcView GIS program (version 3.2a; ESRI, Redlands, CA) to plot the distribution of all individuals, color-coded by leaf margin shape (entire vs. toothed) and sexual system.

RESULTS

Sequence Characteristics

Within Siparuna, the G+C percentages ranged from 56% to 63.8% in ITS-1, 60.7% to 68.8% in ITS-2, and 32.2% to 33.9% in the trnL-trnF intergenic spacer (excluding incompletely sequenced species; Table 2). Gomortega nitida and Glossocalyx longicuspis differed from Siparuna in their ITS-1 and ITS-2 G+C contents (Table 2), but that of the 5.8S rDNA region was constant across taxa. Excluding S. bifida and S. cervicornis, monoecious and dioecious species differed significantly in ITS G+C contents, with monoecious species having higher ratios than dioecious species (61.7–66% vs. 58.5–60.1%; Student's t test, $P < 0.001$). The dioecious S. bifida and S. cervicornis, however, resembled the monoecious species in their G+C content.

Within Siparuna, ITS regions ranged in length from 223 to 240 bp for ITS-1 and from 233 to 244 bp for ITS-2 (Table 2). Gomortega and Glossocalyx had shorter or longer ITS-1 regions than Siparuna. Excluding S. bifida and S. cervicornis, dioecious species had significantly longer ITS-1, and shorter ITS-2 regions than monoecious species (224–240 bp vs. 225–232 bp, $P < 0.01$ for ITS-1; 233–234 bp vs. 238–244 bp, $P < 0.0001$ for ITS-2). Siparuna bifida and S. cervicornis ITS regions resembled those of monoecious species.

Phylogenetic Analyses

The length of the ITS and trnL-trnF alignments, number of phylogenetically informative characters, and tree statistics are summarized in Table 3 (data set is available on the SSB website: http://www.utexas.edu/ftp/dept/systbiol/). The aligned ITS sequences comprised 687 nucleotide positions, of which 165 (26.7%) were parsimony informative. A 36-bp deletion in Glossocalyx and a 13-bp insertion in S. decipiens made part of the alignment difficult. Of the remaining insertions or deletions (indels), 10 were phylogenetically informative in the ingroup: namely, four 1-bp indels in ITS-1, and three 1-bp, two 2-bp, and one 3-bp indels in ITS-2. These indels differentiated monoecious from dioecious species, except that S. bifida and S. cervicornis again grouped with the monoecious sequences (as they had in their G+C content and ITS length).

The trnL-trnF alignment consisted of 372 positions, of which 11 (3%) were parsimony-informative. A 5-bp insertion distinguished most monoecious and dioecious species, except that the dioecious S. bifida and S. cervicornis shared the insertion of the monoecious species.

TABLE 2. Lengths and G C C contents (%) of the ITS and trnL-trnF regions.

	ITS-1		ITS-2		trnL-F	
Species	Length	GC, %	Length	GC, %	Length	GC, %
Glossocalyx						
G. longicuspis	157	59.9	227	55.1	354	32.2
Siparuna						
S. cuspidata	232	60.3	234	64.5	—	30.0[a]
S. cymosa	224	63.8	233	66.1	371	33.4
S. decipiens	240	61.3	233	62.2	—	32.8[a]
S. glycycarpa[b]	232	60.8	233	66.5	—	34.6[a]
S. guianensis	234	63.2	234	68.8	—	33.8[a]
S. aff. monogyna	234	60.3	233	64.8	371	33.4
S. reginae	232	61.6	234	65.0	—	34.4[a]
S. bifida	223	61.4	234	65.4	376	33.0
S. cervicornis	232	61.6	233	64.8	—	33.0[a]
S. aspera	227	56.4	239	61.5	366	33.3
S. conica	225	56.4	239	61.1	—	33.5[a]
S. echinata	225	56.9	239	60.7	365	33.4
S. laurifolia	225	58.7	239	60.7	366	33.9
S. lepidota	225	56.4	239	60.7	366	33.3
S. muricata	225	56.0	240	60.8	366	33.9
S. pauciflora	232	57.8	242	62.4	366	33.1
S. radiata	225	57.8	238	61.3	—	33.2[a]
S. sessiliflora	225	57.8	238	60.9	366	33.3
S. stellulatas	225	56.4	244	61.5	366	33.6
S. Thecaphora	225	56.0	242	60.7	366	33.1
Gomortega						
G. nitida	236	68.6	193	68.4	365	34.0

[a] Denotes G C C contents calculated from partial trnL-trnF sequences.

[b] Bracteanthus glycycarpus Ducke will be transferred into Siparuna in a monograph of the genus (Renner and Hausner, Unpubl. manuscript).

There was no statistically supported conflict among topologies obtained from ITS and trnL-trnF, and the data were therefore combined. Parsimony analysis of the combined sequences resulted in six equally parsimonious trees, all on one island (L D 658, CI D 0.69, excluding uninformative characters, RI D 0.85; Fig. 1 depicts one of them) that showed the African Glossocalyx as sister to a neotropical clade comprising Siparuna and Bracteanthus. (Bracteanthus consisted of a Central Amazonian species, B: glycycarpus, and a second species from Bahîa; both are synonymized and transferred into Siparuna in a monograph of the family [Renner and Hausner, unpubl.].) Within the neotropical clade, a monoecious species, S. decipiens, was sister to all other species, which in turn sorted into a monoecious and a dioecious clade, each with 100% bootstrap support and high decay indices (Fig. 1). There was little resolution within the latter clades except that

TABLE 3. Summary statistics for data matrices and resulting trees from phylogenetic analyses using separate and combined ITS and trnL-trnF sequences of Siparunaceae.

Data partition	No. of characters	No. of variable characters %	No. of parsimony-informative characters	Length of MP trees	No. of trees	CI	RI
ITS-1	263	168 (63.9)	79 (30.0%)	299	63	0.73	0.88
ITS-2	260	164 (63.1)	75 (28.8%)	279	16	0.69	0.84
ITS-1 C ITS-2	523	332 (63.5)	154 (29.4%)	584	6	0.70	0.85
ITS	687	356 (51.8)	165 (26.7%)	619	6	0.70	0.85
trnL-trnF[a]	372	81 (21.8)	11 (3.0%)	155	2	0.78	0.86
ITS C trnL-trnF[a]	1059	437 (41.3)	176 (16.6%)	777	6	0.70	0.85

[a] trnL-trnF sequences were truncated "-27 bp downstream from the spacer’s starting print.

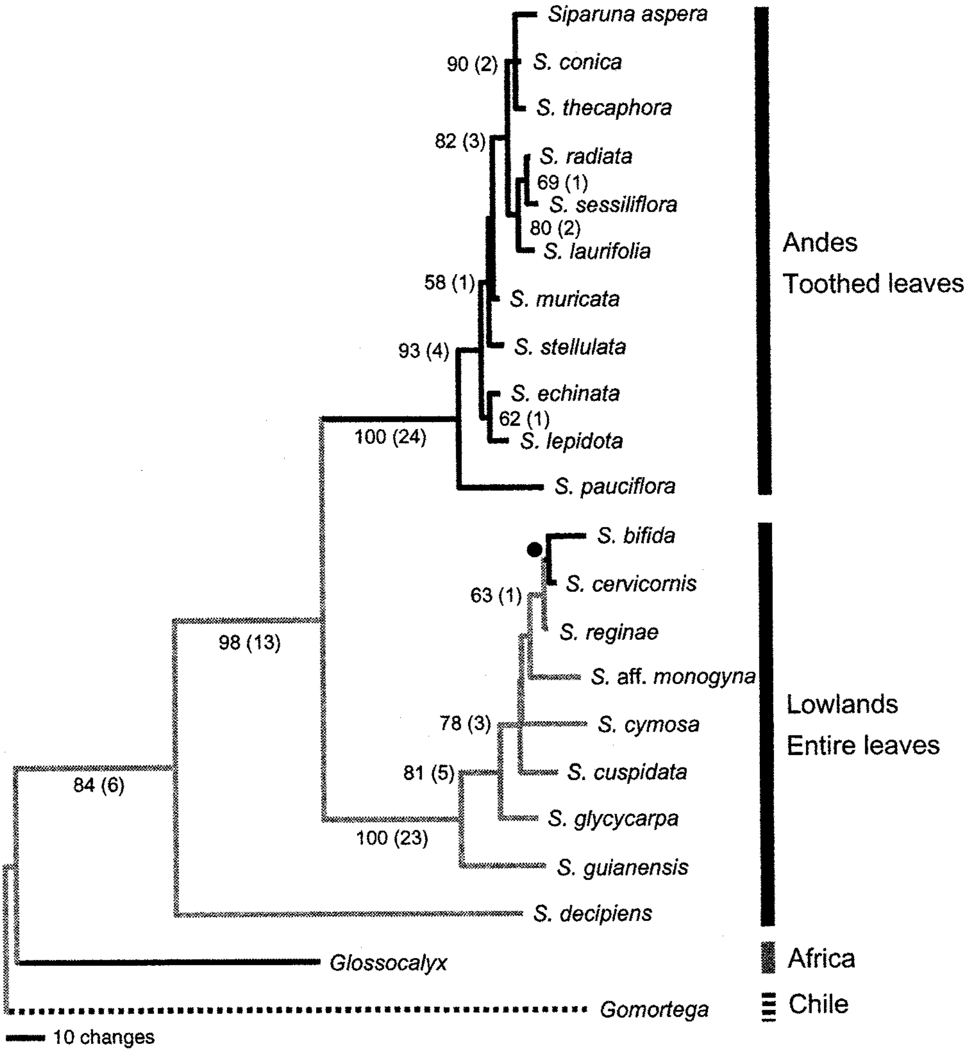

FIGURE 1. One of six equally parsimonious trees resulting from analysis of ITS and trnL-trnF sequences of Siparunaceae (L D 658, CI D 0.69, RI D 0.85). Branch lengths are proportional to numbers of substitutions. Bootstrap support values >50% shown at nodes are based on 1,000 replications ; decay indices follow in parentheses. A node collapsing in the strict consensus is indicated with a solid circle. Dioecious lineages are shown in black, monoecious ones in gray, and prefect-flowered ones dashed. For full species names with authors see Table 1.

S. pauciflora (occurring from Colombia to Nicaragua) was supported as sister to the remaining Andean species and the widespread S. guianensis as sister to the other monoecious species.

ML analysis of the combined data yielded a single most likely topology (not shown) that differed from the parsimony tree in minor rearrangements within the Andean clade. The estimated proportion of invariable sites was 0.34 and the gamma shape parameter was 0.68, indicating that most sites have low substitution rates or are almost invariable, whereas others change at very high rates (Yang and Kumar, 1996).

Molecular Clock Analyses

The likelihoods of the single best nonclock tree (not shown) and single best clock tree (Fig. 2) did not differ significantly (Â^2 D 19.34, df D 21, P < 0.5), and both resembled the parsimony topology except for the mentioned rearrangements among shallowly branched terminal taxa. This justifies the use of sequence divergence in this data set to estimate absolute times from genetic distances

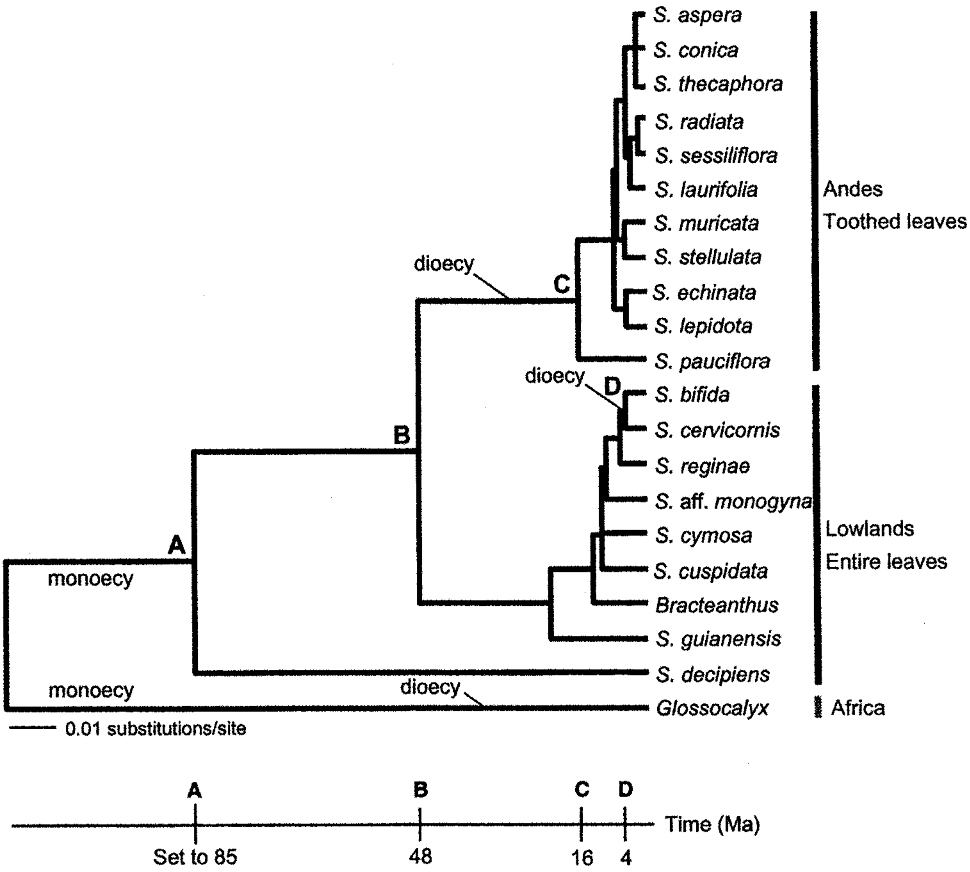

FIGURE 2. Single highest likelihood tree for Siparunaceae found under the GTR C 0 C P_{inv} C clock model. The preferred scenario (three gains of dioecy from monoecy) is illustrated here, based on arguments provided in the text; the sister groups of Siparunaceae have bisexual flowers. Based on either the separation of Africa and South America (complete by 90 Ma) or the oldest fossils of the sister clade of Siparunaceae (86–88 Ma), a minimum age for Siparuna (node A) is 85 Ma. Node B denotes the divergence of the Andean clade, node C the onset of major diversification in the Andean clade, and node D the minimum age of S. bifida and S. cervicornis. All nodes may underestimate ages because of extinction. See text for standard deviations on all estimates.

(i.e., branch lengths measured as ML-GTR C 0 C P_{inv} distances).

To calibrate the genetic distances in the ML tree, we initially planned to use reportedly Siparuna leaves from the Late Eocene (32 Ma) Goshen Flora in Oregon (Chaney and Sanborn, 1933). After Chaney and Sanborn's material was received on loan and compared with extant Siparunaceae and Lauraceae leaves, we determined that their fossils could represent either family. Instead of using fossils to ascertain a minimal age of Siparuna, one can assign an age of 90 Ma to the split between West African Glossocalyx and South American Siparuna, which would be justified if long-distance dispersal between South America and West Africa could be ruled out. Another option is to constrain the age of Siparunaceae with fossils of their sister clade. Gomortega is known only from the Late Oligocene–Early Miocene (24–21 Ma; Nishida et al., 1989), but atherosperms have a long fossil record (reviewed in Renner et al., 2000), the oldest find coming from the Coniacian (86–88 Ma). Based either on tectonics (90 Ma) or on sister group fossils (86–88 Ma), a minimal age for Siparuna (node A in Fig. 2) may be 85 Ma. With node A set to 85 Ma, a substitution rate of 0.001 substitutions per site per million years can be calculated by dividing the genetic distance from node A to the tips (0.08448) by 85. This rate gives a minimum age of 48 § 8 Ma for the divergence of the Andean clade (node B; the distance from node B to the tips is 0.04788 § 0.0075 SD, which, divided by 0.001, gives 48).

A minimum age for the onset of major diversification in the Andean clade (node C) is 16 § 4 Ma (0.01566 § 0.004 SD ¥ 0.001) and a minimum age of S. bifida and S. cervicornis (node D) is 4 § 2 Ma (0.004 § 0.002 SD ¥ 0.001).

Geographical Distribution of Leaf Margin Types and Sexual Systems

Because entire leaf margins typically reflect high mean annual temperatures and rainfall (Wolfe, 1990; Wiemann et al., 1998, and references therein), it is important to consider where the entire-margined leaves and toothed-leaved species occur. Mapping of 6,496 herbarium collections (of all, not just the sequenced species) color-coded by leaf margin type and sexual system showed that toothed leaves typically range into high altitude forests (Fig. 3) and that leaf teeth correlate with dioecy. In contrast, entire-margined species occur in lowland forests and usually are monoecious. The only entire-margined dioecious species are S. bifida and S. cervicornis (shown in red), which occur at altitudes below 700 m in the westernmost Amazon basin. Nineteen of 138 collections of S. bifida come from altitudes between 700 and 1,000 m, but the species is common only in the lowlands (see Table 1 for species' altitudinal ranges).

DISCUSSION

Correlations Among Sexual Systems, Leaf Margins, and Altitude

On the basis of molecular and morphological (leaf margin) data, dioecy appears to have evolved three times in Siparunaceae —once in the ancestor of the Andean siparunas, a second time in the ancestor of the Amazonian lowland species S. bifida and S. cervicornis, and a third time in the African Glossocalyx. In terms of hypothesized character state changes, one other scenario is equally parsimonious. This would invoke dioecy as ancestral in Siparunaceae and as having been lost once in S. decipiens and a second time in the ancestor of the remaining lowland species, followed by reacquisition of dioecy in the lowland S. bifida and S. cervicornis. We prefer the first scenario (three gains) over the second (two losses, followed by a regain) because it is difficult to imagine why dioecy would be lost twice and then regained, all under the same ecological (lowland) conditions.

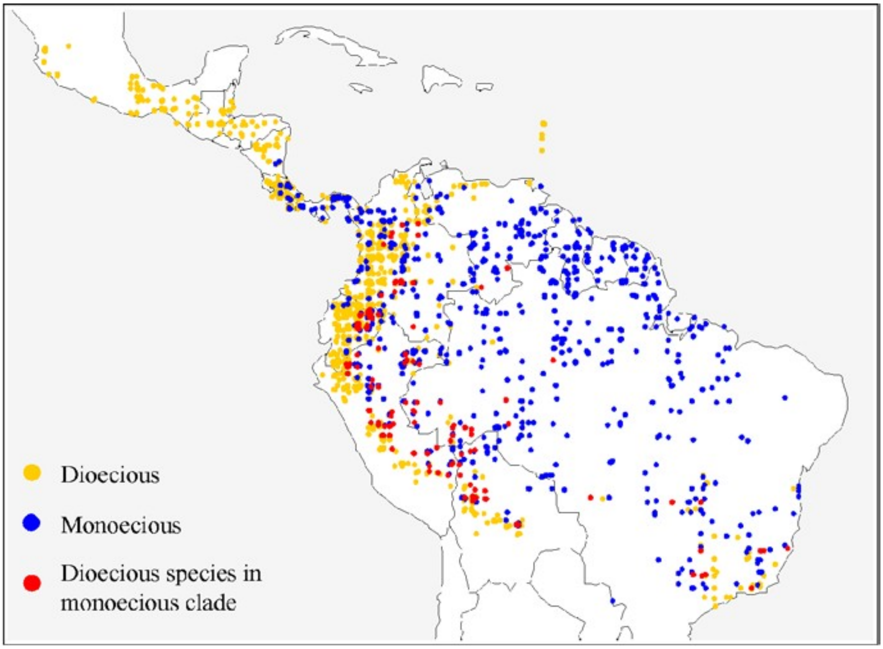

FIGURE 3. The distribution of 6,496 collections of Siparuna, color-coded by mating system. All monoecious species (blue, n D 1,829 collections) have entire leaves and all dioecious species (yellow, n D 4,416 collections) have toothed leaves, except for S. bifida and S. cervicornis (red, n D 251 collections), which are dioecious but have entire leaves.

Under either scenario, dioecy evolved from monoecy in the ancestor of S. bifida and S. cervicornis. The sister group relationship between S. bifida and S. cervicornis seen in the ML tree (Figs. 1, 2) is poorly supported, leaving open the possibility that dioecy arose independently in S. bifida and S. cervicornis. Morphologically, S. bifida and S. cervicornis are extremely similar, differing only in receptacle spinyness and indumentum.

Geographical mapping revealed a strong correlation among altitude, leaf margin shape, and sexual system (Fig. 3). In the neotropics, lowland species have entire-margined leaves and Andean species have toothed leaves. A single toothed species, S. brasiliensis, occurs in the Brazilian Serra do Mar mountains. According to its trnL-trnF sequence, it falls inside the Andean clade (no ITS sequence was obtained; see Material and Methods). The West African Glossocalyx longicuspis has (minutely) toothed leaves and mostly occurs below 700 m. Of the sister taxa of Siparunaceae , Gomortegaceae have entire margins and Atherospermataceae have toothed margins. The ancestor of Glossocalyx and Siparuna could thus have had toothed margins, with teeth being lost in S. decipiens and again in the remaining lowland species. Alternatively, teeth could be a synapomorphy for the Andean dioecious clade and an autapomorphy of Glossocalyx. Because all Andean species as well as Glossocalyx are dioecious, toothed-margins in Siparunacea e correlate completely with dioecy. The converse is not true for entire margins and monoecy, however, because the two lowland species that switched to dioecy (S. bifida and S. cervicornis) retained the entire-margined leaves of their monoecious ancestors. Eight additional monoecious lowland species with entire margins were not sequenced, but their morphological characters (inflorescences, flowers, and indumentum) suggest that they would fall in the entire-margined clade.

Although our results support the well-established correlation between leaf margin shape and mean annual temperature (mediated by altitude; e.g., Wolfe, 1990; Wiemann et al., 1998), they provide no evidence for a correlation between altitude and sexual system, even though the two co-vary. Dioecy evolved once in a lowland clade and once in the ancestor of the Andean clade, and thus no inference is possible about whether lowland or montane conditions selected for dioecy. Other studies indicate that montane conditions sometimes favor dioecy, apparently in connection with changes in the pollinator spectrum (Arroyo and Squeo, 1990; Delph, 1990), a situation that does not apply to Siparuna because all species are pollinated by gall midges (Feil and Renner, 1991; Feil, 1992; Renner et al., 1997; Schulz-Burck, 1997) . Whether the two switches to dioecy in Siparuna happened in response to different selective factors or not, they clearly occurred at different times (Figs. 1, 2). The lowland dioecious species may be 4 Ma old, whereas the Andean dioecious clade may be between 48 and 16 Ma old.

Evolutionary Pathways to Dioecy

In Siparunaceae , dioecy evolved from monoecy, raising the question of how this might have happened . Field and herbarium observations in the dioecious species show that inconsistent males and females are both relatively common (Fouilloy, 1974; Feil, 1992; Renner and Hausner, 1997, and unpubl.). Also, bisexual flowers have never been found among thousands of flowers in living or herbarium material of all species. Together, these two observations suggest that the evolution of dioecy in Siparunacea e has followed the monoecy/paradioecy/dioecy pathway (Lloyd, 1972a,b, 1975, 1980, 1981; Webb, 1999). In this pathway, a monoecious population of plants with genetic variation among individuals with regard to flower sex ratios is invaded by genotypes with severely biased floral sex ratios. Transitional populations are expected to include inconstant males and females and would be classified as paradioecious (see Lloyd, 1980). As discussed by Lloyd, the crucial difference between this pathway and another pathway to dioecy that starts from gynodioecious ancestors lies in the developmental nature of the gender changes. In the potentially bisexual flowers of gynodioecious plants, at least two mutations must become linked to cause male-sterile and female-sterile flowers on different individuals to arrive at dioecy (Lewis, 1942; Lloyd, 1976, 1980; Charlesworth and Charlesworth, 1978; Webb, 1999). Under its original definition, the pathway was seen as starting with male-sterility alleles that abolished pollen production in some proportion of individuals, which then selected for the reciprocal genotype in other individuals. In contrast, the evolution of dioecy from monoecy was envisioned as the spread of alleles affecting individuals' floral sex ratio because male and female sterility was already established. The two pathways were seen as fundamentally different (Lloyd, 1980) and as having played a role in different lineages (Lewis, 1942; Lloyd, 1980; see also Renner and Ricklefs, 1995). The broadening of the term gynodioecy (e.g., Barrett, 1998; Sakai and Weller, 1999; Sarkissian et al., 2001) to cover interbreeding between monoecious and pistillate individuals (rather than just interbreeding between perfect-flowered and pistillate individuals) affects the discussion of the two pathways. As pointed out by Barrett (1998) , gynodioecy under the broader definition grades into paradioecy, the interbreeding of reciprocally sex-biased monoecious plants, at least if pure females are rare. There is a danger, therefore, that workers may be discussing the same thing under two names. For example, under the traditional definition, the variable mixes of monoecious and dioecious plants in populations or species of Leptinella (formerly Cotula; Lloyd, 1972a,b, 1975), Myrica gale (Lloyd, 1981), and Sagittaria latifolia (Sarkissian et al., 2001) would be discussed in terms of the monoecy/paradioecy/dioecy pathway. Under the new definition, the same data are seen as fitting the gynodioecy/dioecy pathway (e.g., for Sagittaria; Sarkissian et al., 2001).

Another difference between the two pathways to dioecy (to our knowledge not discussed by earlier workers) concerns the pollinator/flower interface. Because monoecious species already have unisexual flowers, they have coped with whatever pollinator attraction problems may result from loss of signals or rewards when flowers lose functional pollen, anthers, or pistils (e.g., where pollen was the reward or where nectar came from the pistils as in monocots). Subsequent mutations that merely affect flower sex ratios may have few additional effects on pollinators. In contrast, female-sterile mutants invading a gynodioecious population compete for pollinators with perfect-flowered individuals and may face being avoided by pollinators. In Siparuna, the pollinator reward is an oviposition site. Flowers are closed except for a minute apical hole and offer neither pollen nor nectar. Both monoecious and dioecious species are pollinated by cecidomyiid midges of the genera Asynapta, Clinodiplosis, and Dasineura that oviposit through the flowers' apical hole, whereby they incidentally contact stamens or styles (Feil and Renner, 1991, Feil 1992; Renner et al., 1997; Schulz-Burck, 1997). The midges distribute their numerous eggs onto different flowers and plants, but male flowers always contain more larvae (Feil, 1992; Schulz-Burck, 1997). In monoecious Siparuna, geitonogamy is not prevented by dichogamy (Schulz-Burck, 1997), but nothing is known about self-incompatibility.

Genetic studies suggest that many gender-differentiating mechanisms have been adopted in the angiosperms, with hormone levels as key regulators (Grant, 1999). In the monoecy/paradioecy/dioecy pathway, selection presumably acts on genetic variation in flower production among individuals. Male and female flowers in monoecious species often are produced at widely separate times or places (Lloyd, 1981; Grant, 1999; Webb, 1999; Sarkissian et al., 2001, and references therein). Such spatiotemporal separation is especially apparent in Cucurbitaceae, where alleles that accelerate or delay the onset of male or female flower development have been mutated to synthesize female or dioecious cultivars (George, 1970; Roy and Saran, 1990; Grant, 1999). Generally, plasticity in the timing and spacing of the two flower morphs may allow differential allocation to male and female function depending on plant size and environmental factors (e.g., availabilit y of light or water).

Groups in which the monoecy/paradioecy/dioecy pathway may be important include "-200 angiosperm genera (besides the mentioned Sagittaria, Myrica, and Leptinella), consisting exclusively of monoecious and dioecious species. They are concentrated in Cucurbitaceae, Euphorbiaceae, Moraceae, Myristicaceae, and Urticaceae in the dicots and in Alismatales among monocots. Groups in which the gynodioecy/dioecy pathway may be important are the "-15 genera that contain gynodioecious and dioecious species. (A database of dioecious angiosperm genera with data on pollination, seed dispersal, geographic range, growth form, and breeding systems besides dioecy may be found at http: //www .umsl.edu /"-biosrenn /dioecy. pdf).

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