

Molecular phylogeny and biogeography of the South American genus *Metrodorea* (Rutaceae)

Pedro DIAS^{1*}, Renata Giassi UDULUTSCH², José Rubens PIRANI³

¹School of Arts, Sciences, and Humanities, University of São Paulo, São Paulo, SP, Brazil

²Department of Biological Sciences, Faculty of Science and Letters, University of Estadual Paulista, Assis, SP, Brazil

³Department of Botany, Biosciences Institute, University of São Paulo, São Paulo, SP, Brazil

Received: 30.10.2014

Accepted/Published Online: 16.11.2014

Printed: 30.09.2015

Abstract: The genus *Metrodorea* (Rutaceae), with 6 species, has representatives that vary from small shrubs to tall trees, and its leaf sheaths make it unique in the family; it is restricted to South America, with species diversity concentrated in Brazil. In this study, we applied Bayesian and parsimony methods to infer the molecular phylogeny of *Metrodorea*, and cladistic biogeographic methods to propose an area cladogram for the genus. All results supported *Metrodorea* as monophyletic, and the presence of leaf sheaths is reinforced as its key diagnostic feature. Bayesian and parsimony trees were identical for relationships within *Metrodorea*. The crown clade (*M. flavida*, *M. mollis*) is composed of two species of allopatric distribution and very different morphologies. *Metrodorea* is distributed in three biogeographic subregions: the Amazonian subregion is populated by one species, the Parana subregion by three species, and the Chacoan subregion by five species. Primary Brooks parsimony resulted in only one area cladogram, which differs from the strict consensus tree from the component analysis by the relationships of the Cerrado, and it suggests a closer relationship between Caatinga province and the Amazon subregion.

Key words: Bayesian, parsimony, ITS, trnS-G, Brooks, component, biogeographic regions

1. Introduction

The South American genus *Metrodorea* A.St.-Hil. (Rutaceae) comprises 6 species mostly distributed in Brazil, where all species occur, appearing in 17 out of 26 states. *Metrodorea flavida* K.Krause is the more widespread species, ranging from Suriname and north-central Brazil to Bolivia, throughout the Pantanal biogeographic province in the Amazonian forest, or patches of it, sometimes within other vegetation formations. The remaining five species are distributed mostly in eastern Brazil in Cerrado, Caatinga forest, and Atlantic forest (Figure 1).

Metrodorea is unique in the family Rutaceae for its leaf sheath morphology (Engler, 1931; Kaastra, 1982) (see Figure 2), as both sheaths of each pair of opposite leaves stick to each other until the developing terminal bud forces them to separate. Its representatives vary from small shrubs (*M. mollis* Taub. and *M. concinna* Pirani & P. Dias) to tall trees (*M. flavida* and *M. stipularis* Mart., Figure 2), or both habits within a same species (*M. maracasana* Kaastra and *M. nigra* A.St.-Hil.; Kaastra, 1982; pers. observ.); their leaves are sessile (in *M. concinna* and *M. stipularis*) or petiolate, and 1–3-foliolate, with leaflets sessile (in *M.*

concinna and *M. mollis*) or petiolulate, bearing conspicuous (in *M. mollis*) or inconspicuous glands; their carpels have dorsal apophysis (in *M. mollis*) or not; and their fruits have densely (in *M. flavida*, *M. maracasana*, and *M. mollis*) or sparsely distributed tubercles (Dias et al., 2013).

Taking into account all species of the genus, only *M. nigra* (“carrapateira”, “chupa-ferro” in vernacular Portuguese) has been extensively studied to date, and is one of the species best represented in herbaria. For example, Müller et al. (1995) isolated several compounds from its stems and leaves; Souza et al. (2004) studied its flower and fruit anatomy; Pombal and Morellato (2000) investigated the correlation between its floral traits (color and odor) and main pollinators, as compared to *M. stipularis*; and recent works by Guidugli et al. (2012) and Schwarcz et al. (2010) have been focused on its genetic diversity and structure, and its mating system. In addition, many studies have reported *M. nigra* as a frequent species in floristic surveys (Durigan et al., 2000; Toniato and Oliveira-Filho, 2004; Santos et al., 2007b), playing an important ecological role in tropical deciduous forests (Metzger, 2000), and also as a key species for forest restoration purposes in this biome (Chagas et al., 2004).

* Correspondence: pdias@usp.br

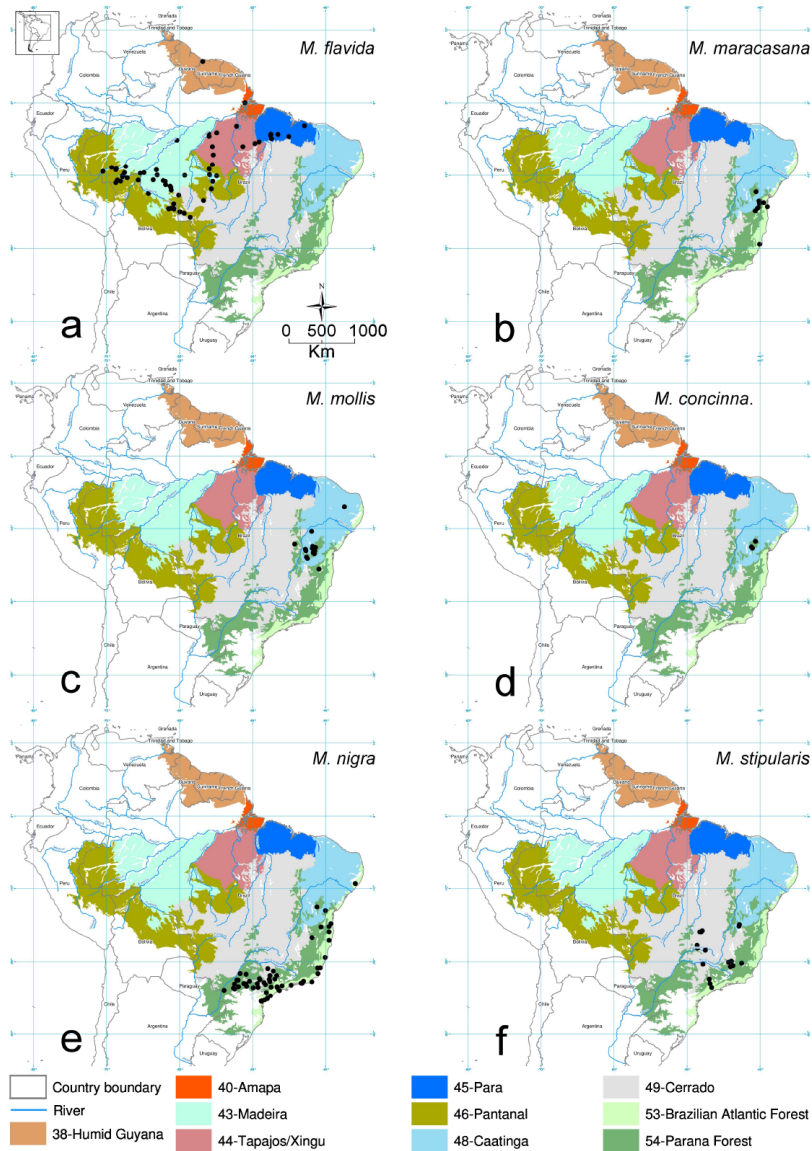


Figure 1. Geographic distribution of *Metrodorea* species. (a) *M. flavida*, (b) *M. maracasana*, (c) *M. mollis*, (d) *M. nigra*, (e) *M. concinna*, and (f) *M. stipularis*. Only biogeographic provinces where *Metrodorea* representatives occur are colored (Morrone, 2006). Provinces Amapa, Humid Guyana, Madeira, Tapajos/Xingu, Pantanal, and Para belong to the Amazonian subregion; provinces Caatinga and Cerrado belong to the Chacoan subregion; and provinces Brazilian Atlantic Forest and Parana Forest belong to the Parana subregion.

From a phylogenetic standpoint, in a previous study of the family by Groppo et al. (2012), *Metrodorea* emerged as sister to *Esenbeckia* Kunth (of the same subtribe Pilocarpinae) within a strongly supported clade composed also of *Balfourodendron* Mello ex Oliv. and *Helietta* Tul., although these two genera were ascribed to a different subfamily by Engler (1931). Regarding the other two genera of the subtribe Pilocarpinae, *Pilocarpus*

Vahl and *Raulinoa* R.S. Cowan, only *Pilocarpus* has already been included in previous phylogenetic works (Groppo et al., 2012), although in these studies *Pilocarpus* emerged within an unsupported group; meanwhile the phylogenetic position of *Raulinoa* remains unknown. In general, the evolutionary relationships considering the whole tribe Galipeae (composed of subtribes Galipeinae and Pilocarpinae) are scarcely studied.

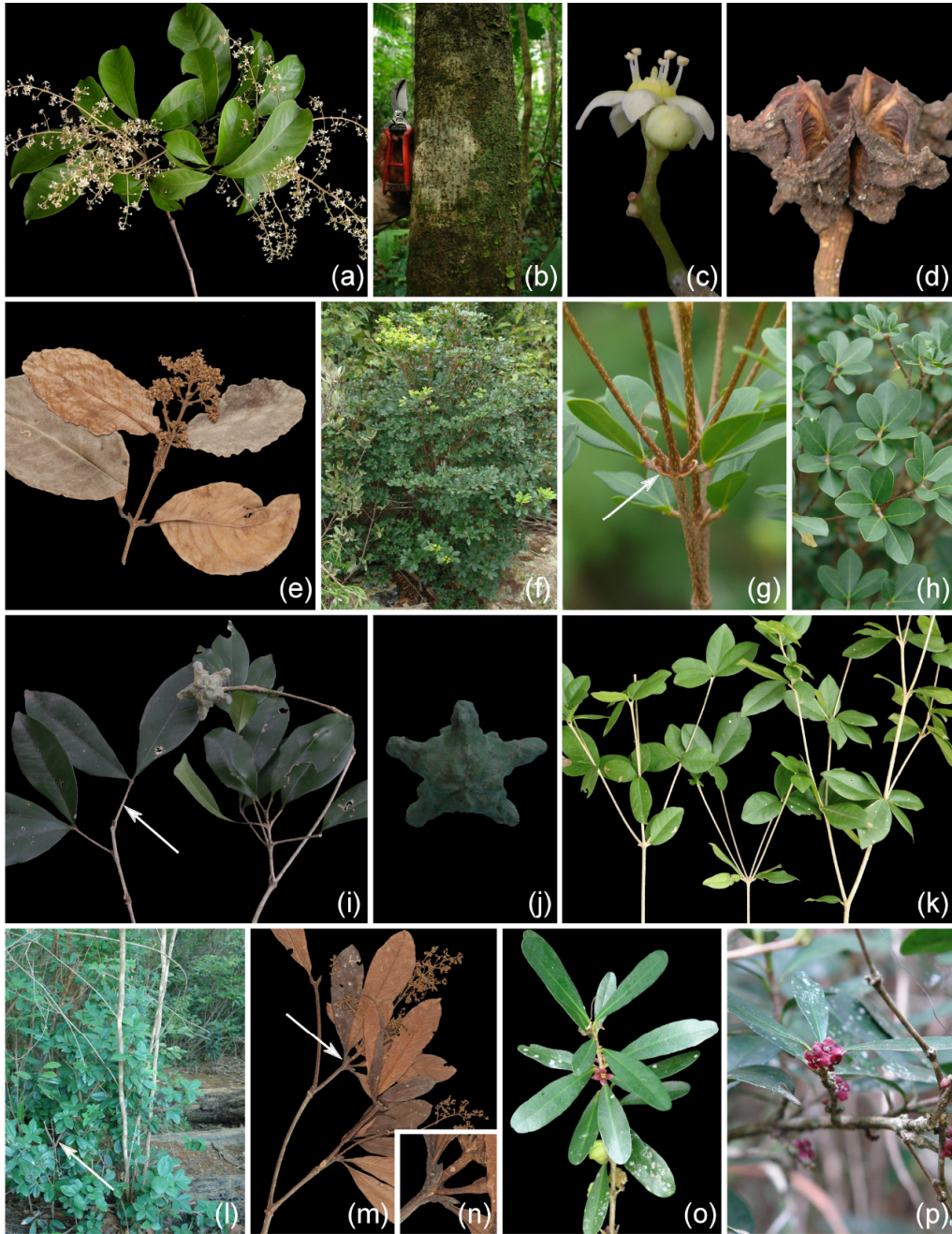


Figure 2. Main vegetative and reproductive characters of *Metrodorea* and *Raulinoa*. *Metrodorea flavida*: a) flowering shoot, b) trunk showing the arborescent habit, c) flower with cream corolla and filaments, and d) fruit with apophyses. *Metrodorea maracasana*: e) flowering shoot with unifoliolate leaves. *Metrodorea mollis*: f) shrubby habit, g) leaf sheath (arrow), and h) leaf blade detail. *Metrodorea nigra*: i) fruiting shoot with petiolate leaves (arrow), j) fruit. *Metrodorea concinna*: k) vegetative shoot with bifoliolate leaves, l) shrubby habit. *Metrodorea stipularis*: m) flowering shoot with sessile leaves (arrow indicates region enlarged and shown in n), n) detail of the leaflets directly connected to the sheath. *Raulinoa echinata*: o) flowering and fruiting shoot with opposite, unifoliolate leaves and tetramerous, vinaceous flower, p) detail of a flowering shoot with 30 buds (a–d from P.Dias 300, e from J.Spada 16/78 [NY00382452], f–h from P.Dias 320, i–j from R.G. Udulutsch 26, k–l from P.Dias 318, m–n from Riedel s/n [NY01038600], o–p from P.Dias 275).

Although the genus *Metrodorea* is clearly characterized by its leaf sheaths, its monophyly is uncertain due to the similar floral morphology shared with *Balfourodendron*, *Esenbeckia*, and *Helietta* (Kaastra, 1982; Pirani, 1998). Moreover, considering that biogeographic studies in plants have largely lacked a phylogenetic component (Fiaschi and Pirani, 2009), the main objectives of this paper are to determine the phylogenetic relationships among *Metrodorea* known species and to propose an area cladogram for the genus, which will be useful for comparative studies of other Neotropical organisms. To do this, we have inferred a molecular phylogeny of the genus based on ITS (nrDNA) and *trnS*-G (cpDNA) spacers, and performed a cladistic analysis of its historical biogeography.

2. Methods

2.1. Taxon sampling

Plant samples used and voucher information are presented in Table 1. All 6 species of *Metrodorea* were included in our analyses. Out-groups (Nixon and Carpenter, 1993) were selected based on previous studies (Engler, 1931; Groppo et al., 2012) from *i*) all other genera of the same subtribe Pilocarpinae (*Esenbeckia*, *Pilocarpus*, and *Raulinoa*), *ii*) a genus of another subtribe (Galipeinae) in the same tribe Galipeae (*Galipea*), and *iii*) three genera of other subfamilies (*Balfourodendron*, *Helietta*, and *Zanthoxylum*). We included one species for each genus represented in out-

groups based on previous studies (Engler, 1931; Groppo et al., 2012). All trees were rooted with *Zanthoxylum*.

2.2. DNA extraction, amplification, and sequencing

DNA was extracted from leaves dried in silica-gel using the DNeasy Plant Minikit (QiaGen), according to the manufacturer's protocol.

We used the nuclear ribosomal spacers ITS1 and ITS2, and the gene 5.8S (hereafter referred to collectively as ITS) and the cpDNA *trnS*-G intergenic spacer.

ITS was amplified using the primers TATGCTTAAAYTCAGCGGGT and CCTTATCATTTA-GAGGAAGGAG (developed by Kenneth Wurdack), according to the protocol of Stanford et al. (2000). For the *trnS*-G sequences, we used the primers and the conditions described by Hamilton (1999), except that we increased the extension time by 3 s. All PCR products were purified with the QIAQuick PCR Purification Kit (QiaGen). ITS amplified products were run in 1% agarose gel to corroborate the existence of a single band (e.g., Álvarez and Wendel, 2003). Sequences were generated using an ABI 377 automatic sequencer with the same pairs of primers.

All sequences were analyzed and assembled with the phred/phrap/consed (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998) packages together with perl scripts developed by the first author (available upon request) as follows: original sequences were blasted with the checkGB script (this study) to assure that they were

Table 1. List of taxa included in the phylogenetic analyses of *Metrodorea* and relatives with voucher information relative to collector name and herbaria (acronyms according to Holmgren et al., 1990), and GenBank accession numbers for ITS and *trnS*-G sequences obtained in this study.

Taxon	Collector #	Herbarium	GenBank #: ITS/ <i>trnS</i> -G
<i>Balfourodendron riedelianum</i> (Engl.) Engl.	P. Dias 217	SPF	[GenBank:KC502921/KC502934]
<i>Esenbeckia pumila</i> Pohl	P. Dias 225	SPF	[GenBank:KC502922/KC502935]
<i>Galipea trifoliata</i> Aubl.	P. Dias 230	SPF	[GenBank:KC502923/KC502936]
<i>Helietta puberula</i> R.E. Fr.	P. Dias 278	SPF	[GenBank:KC502924/KC502937]
<i>Metrodorea flavida</i> K. Krause	P. Dias 229	SPF	[GenBank:KC502925/KC502939]
<i>M. maracasana</i> Kaastra	L.C. Senra 53	SPF	[GenBank:KC502926/KC502940]
<i>M. mollis</i> Taub.	P. Dias 320	SPF	[GenBank:KC502927/KC502941]
<i>M. nigra</i> A.St.-Hil.	P. Dias 264	SPF	[GenBank:KC502928/KC502942]
<i>M. concinna</i> Pirani & P.Dias	P. Dias 318	SPF	[GenBank:KC502929/KC502938]
<i>M. stipularis</i> Mart.	P. Dias 263	SPF	[GenBank:KC502930/KC502943]
<i>Pilocarpus jaborandi</i> Holmes	P. Dias 252	SPF	[GenBank:KC502931/KC502944]
<i>Raulinoa echinata</i> R.S. Cowan	P. Dias 257	SPF	[GenBank:KC502932/KC502945]
<i>Zanthoxylum rhoifolium</i> Lam.	P. Dias 232	SPF	[GenBank:KC502933/KC502946]

not from contaminants; after confirmation, direct and reverse sequences were analyzed and assembled with the phred/phrap/consed packages (phred to assign a quality value to each called base, phrap to assemble and create contig sequences, and consed to visualize and edit the final sequences), and finally regions of each sequence were analyzed with the script phred20 (this study) before submitting consensus sequences to multiple sequence alignment.

2.3. Alignment and tree searches

We used the following approaches: 1) sequences were aligned with the ClustalX (Thompson et al., 1997) software setting accurate pairwise parameters with default values before multiple alignment, and then the multiple alignment was trimmed based on the shortest sequence with MrBayes' (Ronquist and Huelsenbeck, 2003) command "exclude"; and 2) same as 1 (above) for multiple sequence alignment, but we used PAUP* (Swofford, 2002) with gap as a fifth state (Giribet and Wheeler, 1999; Ogden and Rosenberg, 2007), and applied parsimony as optimality criterion. The incongruence length difference (ILD, Farris et al., 1995) was used to test the heterogeneity of partitions with 1000 replicates and $P \leq 0.01$ conducted in PAUP* (Swofford, 2002). We treated ITS and *trnS-G* as different partitions.

Bayesian searches were conducted according to GTR + Γ + I model, which was selected as the optimal model for both partitions independently in MrModelTest performed through the hierarchical likelihood ratio test (hLRT, Huelsenbeck and Crandall, 1997). Tree searches were performed using a cvs parallel version of MrBayes (Ronquist and Huelsenbeck, 2003) on a Linux cluster using Markov chain Monte Carlo (MCMC) methods. We executed 4 parallel runs, each with 4 simultaneous chains (1 heated and 3 cold), a total of 16 chains, for 100 million generations, sampled at every 1000th generation, and the burn-in (5 million generations) was estimated by means of the shrink factor with the coda package in R (R Development Core Team, 2014). An all-compatible consensus tree was computed from the remaining 95,000 posterior probability saved trees.

Clades with posterior probability support values (PPS) of 90%–100% were assumed as moderately to strongly supported.

Maximum parsimony (Fitch, 1971) analyses were based on the branch-and-bound algorithm (Hendy and Penny, 1982) and the 'MulTrees' option in effect. Branch support was evaluated by mean of decay analysis (DI, Bremer, 1988) with the AutoDecay software and nonparametric bootstrap (BS, Felsenstein, 1985), using 1 million replicates and the same procedure applied in tree search.

For support estimates, we adopted moderately ($\geq 75\%$ to $< 95\%$) to strongly supported ($\geq 95\%$) branches.

2.4. Geographic distribution and historical biogeography

Distribution range data were taken from herbaria, the literature (mostly from Kaastra, 1982), and our own collections. When latitude and longitude coordinates were not available, approximate retrospective georeferencing was performed using the NIMA's gazetteer database (USBG, 1963–onwards) together with a search script developed by the first author (this study), and posterior visual inspection. Distribution maps were assembled with ArcGis (ESRI) and the Digital Basemap of the Americas (Bletter et al., 2004). Areas of endemism were named according to the classification of Morrone (2006) for the Neotropical region, and the units adopted with modifications in the case of Pantanal and Madeira provinces based mainly on Olson et al. (2001) and topographical data.

Historical biogeographic analyses were conducted based on the distribution and phylogeny of *Metrodorea* species and distributional data from the literature. Primary Brooks parsimony analysis (BPA) (Wiley, 1987; Veller and Brooks, 2001) and component analysis (Nelson and Platnick, 1981) were conducted to infer area relationships. BPA analysis was performed with PAUP* (Swofford, 2002) using a branch-and-bound search, and component analysis was performed with Component (Page, 1993) using a heuristic search with subtree-pruning-and-regrafting branch swapping. Data matrix construction and tree handling were performed with Mesquite (Maddison and Maddison, 2014) and MacClade (Maddison and Maddison, 2003).

3. Results

3.1. Phylogenetic analyses

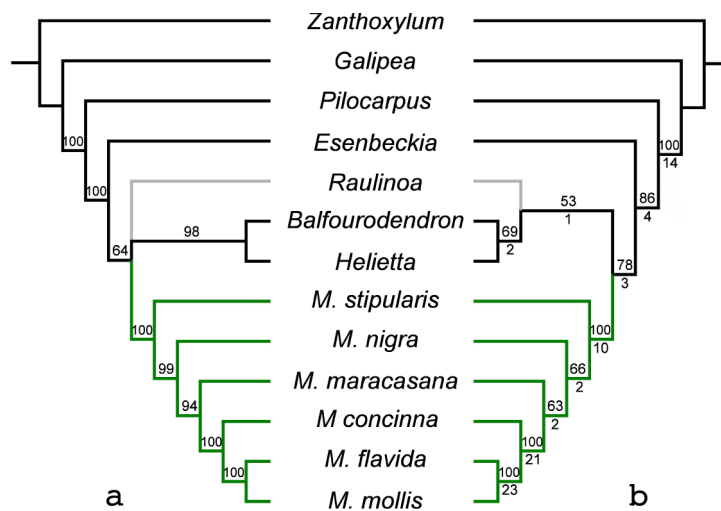
After performing sequence quality analyses and trimming, we obtained an ITS aligned matrix with 628 characters and a *trnS-G* matrix with 696 characters, which were used in all analyses. Table 2 presents a summary of data matrices used in all tree searches.

ITS and *trnS-G* analyses gave different results in terms of resolution, but not incongruent phylogenetic signals. ITS is uninformative; there is a polytomy within *Metrodorea*, although the trees do not contradict each other. Bayesian (BT) and maximum parsimony (MPT, only one tree was recovered by PAUP*) trees based on combined ITS and *trnS-G* matrices differ only by the relative position of *Raulinoa echinata* Cowan (gray branches in Figure 3). In MPT, *Raulinoa* appears with (*Raulinoa*, (*Balfourodendron*, *Helietta*)) in a weakly supported (BS = 54%, DI = 1) clade, meanwhile *Raulinoa* in BT remains in a polytomy (Figure 3).

Only two out-group branches show weak support (BS = 53% and 69%), two branches are moderately supported (BS = 78% and 86%), and one is strongly supported (BS = 100%), as well as *Metrodorea* (BS = 100%, DI = 10).

Table 2. Tree statistics summary of the partitions (ITS and *trnS*-G) used in the Bayesian and parsimony searches.

Characteristic	Partition	
	ITS	<i>trnS</i> -G
Aligned length	628	696
Variable sites (proportion)	36.62	35.2
Parsimony informative sites (proportion)	9.71	14.22
Indel (proportion)	8.12	12.5
Substitution model (Bayesian only)	GTR + Γ + I	GTR + Γ + I

**Figure 3.** Phylogenetic trees of *Metrodorea*. Bayesian tree (a), maximum parsimony bootstrap tree (b). Numbers above branches represent proportion of posterior probability (a) or bootstrap values (b). Numbers below branches represent decay values (b). Green color indicates the genus *Metrodorea* and grey color emphasizes *Raulinoa*.

The monophyly of *Metrodorea* has been corroborated in all analyses (PPS and BS = 100%, Figure 3). The first dichotomy splits *M. stipularis* from the other 5 species, which in turn constitute a group supported on BT (PPS = 99%) and MPT (BS = 66%, and DI = 2). In the group formed by the 5 remaining species of *Metrodorea*, *M. nigra* is sister to all other species. The clade (*M. maracasana*, (*M. concinna*, (*M. flavida*, *M. mollis*))) is moderately supported on both BT (PPS = 94%) and MPT (BS = 63%, DI = 2). The monophyletic group comprising *M. concinna*, *M. mollis*, and *M. flavida* is strongly supported on BT (PP = 100%) and MPT (BS = 100%, DI = 21). The crown clade (*M. flavida*, *M. mollis*) is strongly supported on all trees (PP and BS = 100%, DI = 23).

3.2. Geographic distribution and historical biogeography

The genus *Metrodorea* is distributed in 10 provinces of 3 subregions (Figure 1): *i*) the Amazonian subregion includes the provinces Amapa, Humid Guyana, Madeira, Pantanal, Para, and Tapajós-Xingu, *ii*) the Chacoan subregion includes the provinces of Caatinga and Cerrado, and *iii*) the Parana subregion includes Brazilian Atlantic Forest and Parana Forest provinces. Of these biogeographic areas, the Amazonian subregion is populated only by *M. flavida*, whereas in the Parana subregion three species occur (*M. maracasana*, *M. nigra*, and *M. stipularis*), and the Chacoan subregion contains 5 species (those that occur in the Parana subregion plus *M. mollis* and *M. concinna*).

Primary Brooks parsimony analysis (Wiley, 1987; Veller and Brooks, 2001) for *Metrodorea* resulted in only one area cladogram, which differs from the strict consensus tree from the component analysis (Nelson and Platnick, 1981) by the relationships of the Cerrado (Figure 4).

4. Discussion

4.1. Phylogenetic analyses

The results obtained in this work show that *Metrodorea* is a strongly supported monophyletic group (Figure 3), which is in agreement with previous, noncladistic suggestions that emphasized the morphological peculiarity of the genus (Kaastra, 1982).

Raulinoa is endemic to Santa Catarina state (southern Brazil), and is known to occur only near the margins of the Itajaí-Açu river, and its distinctive morphology includes the presence of spines on branches, unifoliolate opposite leaves, and tetramerous flowers with purplish corolla (Figure 2). This is the first time *Raulinoa* has been included in a phylogenetic analysis, although its precise relationships

to other genera need to be further investigated.

Our molecular results support the peculiar leaf sheath (Figure 2) as a key diagnostic feature (and likely synapomorphy) of the genus. Although BT differs from MPT by the relative position of *Raulinoa echinata*, the internal relationships in *Metrodorea* are identical.

In the studies by Groppo et al. (2012), although focused mainly on subfamily and tribe levels, only one species of *Metrodorea* was included and it emerged as sister to *Esenbeckia*, and conformed a clade with the other two sister genera *Balfourodendron* and *Heliotta*. In the present study, however, *Esenbeckia* was not found as sister to *Metrodorea*. In any tree where the sister-group relationship of *Metrodorea* is clear (Figure 3), *Esenbeckia* is sister to *Raulinoa* or to the clade ((*Raulinoa*, (*Balfourodendron*, *Heliotta*)).

In the genus *Metrodorea*, the basal position of *M. stipularis* corresponds with the only species with sessile leaves (Figure 2), indicating that this morphological feature evolved exclusively in this species of the genus, as all other terminals (out-groups included) have petiolate

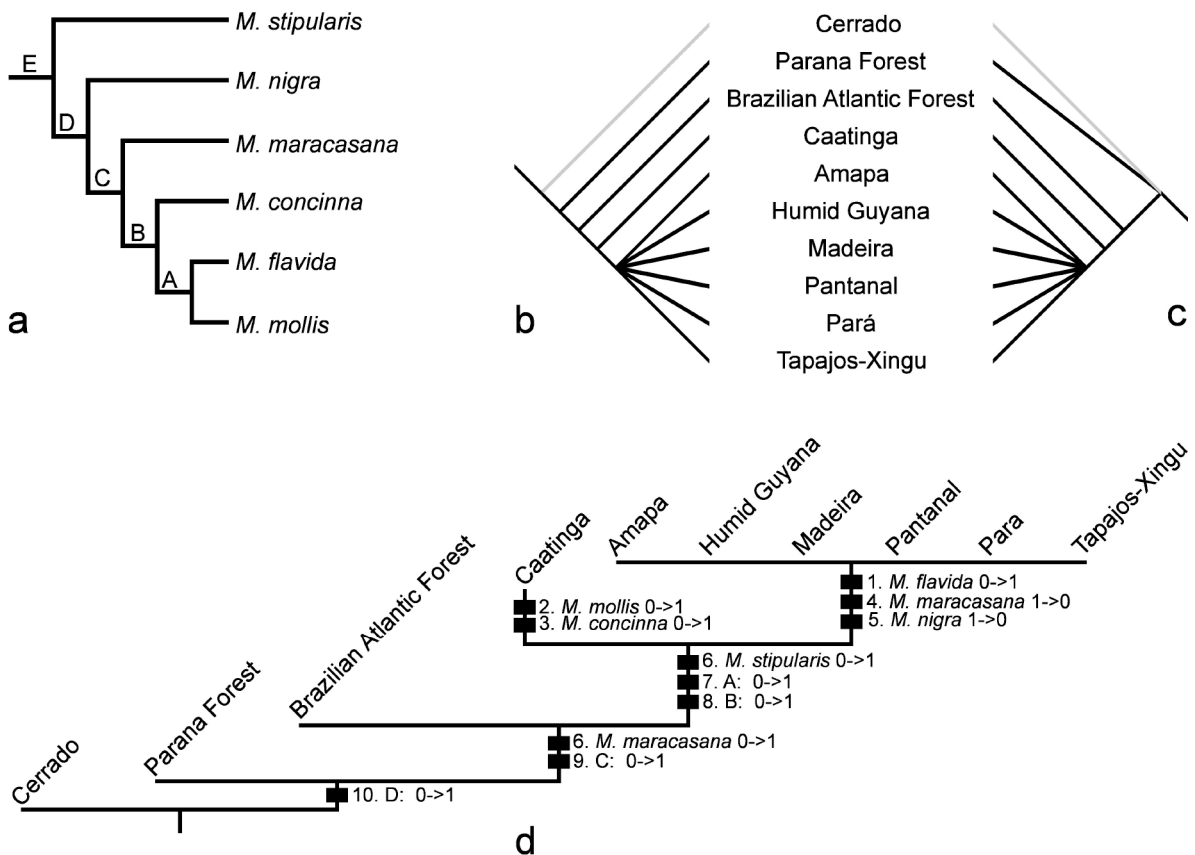


Figure 4. Area cladograms of *Metrodorea*. (a) Phylogeny of *Metrodorea* with components used in the cladistic biogeographic analyses, (b) Brooks parsimony analysis, (c) strict consensus tree from the component analysis (note that (b) and (c) differ only by the relative position of Cerrado province, in grey) and (d) Brooks parsimony analysis tree with species in both (a) and (d), capital letters mean components of the *Metrodorea* phylogeny.

leaves. In his key to the genus, Kaastra (1982) used this character (petioles completely adnate to the sheaths) to distinguish *M. stipularis* from the remaining species of the genus. Because the anatomical nature of the petiole-sheath relationship is still unknown, we considered the leaves to be sessile, i.e. the leaflets to be directly connected to the leaf sheath (Figure 2).

In the clade with petiolate leaves, *M. nigra* is sister to all other species. *Metrodorea nigra* is a common tree species, with purplish (occasionally cream-colored) flowers, found in (semi-) deciduous and Atlantic forests from northeastern (Piauí) to southern (Paraná) Brazil (Figure 1).

The remaining four species are restricted to the Amazon (*M. flavida*) or Caatinga (*M. maracasana*, *M. mollis*, and *M. concinna*) regions (but see discussion below) and morphologically are recognized by having lobed disks (although *M. stipularis* also has lobed disks, it is differentiated from the other species of the genus by its sessile leaves).

Metrodorea maracasana, a tree with uniformly 1-foliolate leaves found only in southeastern Bahia (northeastern Brazil) and northern Espírito Santo (southeastern Brazil), is sister to the other 3 species (*M. concinna*, *M. mollis*, and *M. flavida*) of the “Amazon–Caatinga” clade. Both *M. concinna* and *M. mollis* are shrubs (Figure 2) found only in Caatinga (Figures 1), whereas *M. flavida* is a tall tree (Figure 2) that occurs in the Amazon (Figure 1). Furthermore, the capsules of *M. concinna* are sparsely tuberculate, while those of the crown the clade (*M. flavida*, *M. mollis*) are densely tuberculate. Moreover, of these 3 species, the leaf blade of only *M. mollis* has dark black, conspicuous glands on the abaxial surface. The species of the crown clade (*M. flavida* and *M. mollis*) are very different from each other; *M. mollis* is a shrub with sessile leaflets with dark glands on the abaxial surface of the blade, whereas *M. flavida* is a tall tree with petiolulate leaflets without conspicuous glands on the abaxial surface of the blade. These two species also are allopatric, and more strikingly they are endemic to different biogeographic subregions of the Neotropical region (Figures 1): *M. mollis* is restricted to the Caatinga province of the Chacoan subregion and *M. flavida* is endemic to the Amazonian subregion.

4.2. Geographic distribution and historical biogeography

Metrodorea is restricted to the Neotropical region of South America (Figure 1), where it occurs in all major biomes (according to the classification of Woodward, 2009) in tropical rain forests (Amazon forest and Atlantic forest), wetlands (in the west), and tropical seasonal forests (mainly in the southeast and south), and the distributions of most species overlap in northeastern Brazil (Figure 1). In this country, while the genus occurs in several vegetation formations and biogeographic provinces, 5 of

its 6 species are found in Caatinga, and more specifically in the northeastern state of Bahia.

According to Morrone’s system of biogeographic units (Morrone, 2006), the Neotropical region comprises 4 subregions: the Caribbean subregion (24 provinces), the Amazonian subregion (13 provinces), the Chacoan subregion (4 provinces), and the Parana subregion (3 provinces).

Although the core distribution of the genus *Metrodorea* is in the Chacoan subregion (Figure 1), the genus is far from well represented in the different provinces of this subregion, as 4 out of 6 species were collected in the Caatinga province (*M. maracasana*, *M. mollis*, *M. concinna*, and *M. nigra*), 2 in the Cerrado province (*M. nigra* and *M. stipularis*), and none in the Chaco and Pampa provinces.

Metrodorea stipularis, sister to all other species of the genus, is distributed mainly in southeastern Brazil (Minas Gerais and São Paulo states, Figure 1); its distribution is concentrated in the Parana Forest province, with some individuals collected in the Cerrado province (Figure 1) in patches or peninsulas of the Parana Forest within the Cerrado province.

Metrodorea nigra is sister to the clade composed of *M. maracasana*, *M. concinna*, *M. mollis*, and *M. nigra* (Figure 3), and has the second widest areal distribution (after *M. flavida*), extending from Alagoas southward to Paraná and westward to Minas Gerais (Figure 1). *M. nigra* occurs primarily in the Brazilian Atlantic Forest and Parana Forest provinces. A closer look at the records from both Caatinga and Cerrado provinces showed that, as in the case of *M. stipularis* for Cerrado collections, the specimens were collected in “fragments” or extensions of the Parana Forest province in the Caatinga province (Figure 1).

Metrodorea maracasana, which is sister to the clade ((*M. flavida*, *M. mollis*), *M. concinna*), occurs primarily in the Caatinga province in Bahia state (northeastern Brazil), and only one specimen was collected in the Brazilian Atlantic Forest province in Espírito Santo (southeastern Brazil) (Figure 1). All specimens of this species were collected in fragments or extensions of the Parana Forest within both the Caatinga and the Brazilian Atlantic Forest provinces.

Metrodorea concinna, sister to the crown clade (*M. flavida*, *M. mollis*), is known only from Bahia state (northeastern Brazil) and is restricted to Parana Forest areas within the Caatinga province (Figure 1). The two species that comprise the crown clade, *M. flavida* and *M. mollis*, have allopatric distributions. *Metrodorea flavida* is the only species occurring in, and restricted to, the Amazon subregion (mostly northern Brazil), where it is widely distributed, occurring in the provinces of Amapá, Humid Guyana, Madeira, Pantanal, Para, and Tapajós-Xingu (Figure 1), whereas *M. mollis* is restricted to the Caatinga

province of the Chacoan subregion in northeastern Brazil (Figure 1).

In the BPA tree (Figure 4) the Cerrado province is basal, whereas on the strict consensus tree from the component analysis it fell into a polytomy together with the Parana Forest province and the group composed of all other areas (Figure 4). In the BPA tree, the first split resulted in the separation of Cerrado, where only *M. stipularis* and *M. nigra* are found, from the group of all other areas (Figures 1 and 4). As mentioned above, however, *M. stipularis* and *M. nigra* do not occur in the Cerrado province but in enclaves of the Parana Forest within the Cerrado. In fact, *M. stipularis* is endemic to the Parana Forest, and *M. nigra* occurs in both the Parana Forest and the Brazilian Atlantic Forest. The presence of *M. stipularis* and *M. nigra* in the Parana Forest provides support for the ancient relationship between the Parana Forest and the other provinces.

In the next split, the Parana Forest is separated from the group composed of the Brazilian Atlantic Forest, Caatinga, and the provinces of the Amazonian subregion (Amapa, Humid Guyana, Madeira, Pantanal, Para, and Tapajos-Xingu, and this relationship is supported by the occurrence of *M. stipularis* and *M. nigra*, Figure 4).

The relationship of the Brazilian Atlantic Forest to the group Caatinga + Amazonian subregion is supported by the distribution of *M. nigra* (see occurrence of the D component, with later reversal of *M. nigra* on the Amazonian branch, Figure 4). Furthermore, although *M. maracasana* appears to be distributed primarily in the Caatinga province, it actually occurs in sections of Parana Forest inside that province. Thus, *M. nigra* is the only species occurring in both the Parana Forest and the Brazilian Atlantic Forest provinces, and *M. maracasana* is endemic to the Parana Forest.

References

- Álvarez I, Wendel JF (2003). Ribosomal ITS sequences and plant phylogenetic inference. *Mol Phylogenet Evol* 29: 417–434.
- Bletter N, Janovec J, Brosi B, Daly DC (2004). A digital basemap for studying the Neotropical flora. *Taxon* 53: 469–477.
- Bremer K (1988). The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* 42: 795–803.
- Chagas RK, Durigan G, Contieri WA, Saito M (2004). Crescimento diametral de espécies arbóreas em Floresta Estacional Semidecidual ao longo de seis anos. In: Bôas OV, Durigan G, editors. Pesquisas em conservação e recuperação ambiental no Oeste Paulista: resultados da cooperação Brasil/Japão. São Paulo: Páginas & Letras, pp. 265–290 (in Portuguese).
- Dias P, Udulutsch RG, Pirani JR (2013). A new species of *Metrodorea* (Rutaceae) from Brazil: morphology, molecular phylogenetics, and distribution. *Phytotaxa* 117: 35–41.
- Durigan G, Franco GADC, Saito M, Baitello JB (2000). Structure and diversity of the arboreal component of the forest at Caetetus Ecological Station, Gália, SP. *Rev Bras Bot* 23: 371–383 (article in Portuguese with an abstract in English).
- Engler HGA (1931). Rutaceae. In: Engler HGA, Prantl K, editors. *Die Natürlichen Pflanzenfamilien*. 2nd ed. Vol. 19a. Leipzig, Germany: Wilhelm Engelmann, pp. 187–359.
- Ewing B, Green P (1998). Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* 8: 186–194.
- Ewing B, Hillier L, Wendl MC, Green P (1998). Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 8: 175–185.
- Farris JS, Källersjö M, Kluge AG, Bult C (1995). Testing significance of incongruence. *Cladistics* 10: 315–319.
- Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.

The relationship among Brazilian Atlantic Forest, Caatinga, and Amazonia has been discussed in a previous study by Santos et al. (2007a), based on raw distributional data of tree species, where it is suggested that some areas inside the Caatinga are more strictly related to the Amazon forest than to the Brazilian Atlantic Forest.

Our results clearly demonstrate the monophyly of the genus *Metrodorea*, and its distinctive morphology further strengthens that status, and suggest that the Amazon subregion is historically more closely related to the Caatinga province.

Acknowledgments

The authors are grateful to the curators of F, IAN, INPA, MBM, MG, NY, and US for loans of herbarium specimens; to IBAMA for providing the collecting license N. 080/2005-COMON; and to Jacquelyn A. Kallunki for reading an earlier version of this manuscript and many helpful suggestions. We are deeply indebted to David Gordon, Phill Green, and Brent Ewing for kindly providing copies of the phred/phrap/consed packages and associated programs. We also truly thank the two anonymous reviewers and the editor (Pilar Catalan) for excellent suggestions that greatly improved the manuscript. PD also wishes to express his gratitude to Mariana Cabral de Oliveira for guiding him during his first steps in molecular biology at her lab and to Kenneth M Cameron and Jacquelyn A Kallunki for their invaluable support at the Pfizer Plant Research Laboratory of the New York Botanical Garden. This work was partially supported by FAPESP (Procs. 02/09762-6 to PD, 04/15141-0 & 14/18002-2 to JRP), CNPq (Proc. 140945/2004-0 to RGU & 304726/2003-6 to JRP), and the NYBG Virtual Herbarium Project. This study is part of the PhD dissertation of the first author.

- Fiaschi P, Pirani JR (2009). Review of plant biogeographic studies in Brazil. *J Syst Evol* 47: 477–496.
- Fitch WM (1971). Toward defining the course of evolution: minimal change for a specific tree topology. *Syst Zool* 20: 406–416.
- Giribet G, Wheeler WC (1999). On gaps. *Mol Phylogenet Evol* 13: 132–143.
- Gordon D, Abajian C, Green P (1998). Consed: a graphical tool for sequence finishing. *Genome Res* 8: 195–202.
- Gropo M, Kallunki JA, Pirani JR, Antonelli A (2012). Chilean *Pitavia* more closely related to Oceania and Old World Rutaceae than to Neotropical groups: evidence from two cpDNA non-coding regions, with a new subfamilial classification of the family. *Phytokeys* 19: 9–29.
- Guidugli MC, Ferreira-Ramos R, Sousa ACB de, Cidade FW, Marconi TG, Mestriner MA, Gropo M, Alzate-Marin AL (2012). Genetic diversity of *Metrodorea nigra* (Rutaceae) from a small forest remnant in Brazil assessed with microsatellite markers. *Genetics Mol Res* 11: 10–16.
- Hamilton MB (1999). Four primer pair for the amplification of chloroplast intergenic regions with interspecific variation. *Mol Ecol* 8: 513–525.
- Hendy MD, Penny D (1982). Branch and bound algorithms to determine minimal evolutionary trees. *Math Biosci* 60: 133–142.
- Holmgren PK, Holmgren NH, Barnett LC (1990). Part I: The Herbaria of the World [Regnum Veg. vol. 120]. New York: The New York Botanical Garden. Website: <http://sweetgum.nybg.org/ih/> [accessed 2 January 2014].
- Huelsenbeck JP, Crandall KA (1997). Phylogeny estimation and hypothesis testing using maximum likelihood. *Annu Rev Ecol Syst* 28: 437–466.
- Kaastra RC (1982). A monograph of the Pilocarpaceae (Rutaceae). New York, NY, USA: The New York Botanical Garden [Flora Neotropica Monographs].
- Maddison WP, Maddison DR (2003). *MacClade 4*. Sunderland, MA, USA: Sinauer Associates.
- Maddison WP, Maddison DR (2014). Mesquite: a modular system for evolutionary analysis. v. 2.75. Website: <http://mesquiteproject.org> [accessed 2 January 2014].
- Metzger JP (2000). Tree functional group richness and landscape structure in a Brazilian tropical fragmented landscape. *Ecol Appl* 10: 1147–1161.
- Morrone JJ (2006). Biogeographic areas and transition zones of Latin America and the Caribbean islands based on panbiogeographic and cladistic analyses of the entomofauna. *Annu Rev Entomol* 51: 467–494.
- Müller AH, Vieira PC, Silva MF da G. F. da, Fernandes JB (1995). Dihydrochalcones, coumarins and alkaloids from *Metrodorea nigra*. *Phytochemistry* 40: 1797–1800.
- Nelson GJ, Platnick N (1981). *Systematics and biogeography: cladistics and vicariance*. New York, NY, USA: Columbia University Press.
- Nixon KC, Carpenter JM (1993). On outgroups. *Cladistics* 9: 413–426.
- Ogden TH, Rosenberg MS (2007). How should gaps be treated in parsimony? A comparison of approaches using simulation. *Mol Phylogenet Evol* 42: 817–826.
- Olson DM, Dinerstein E, Wikramanayake ED, Burgess ND, Powell GVN, Underwood EC, D'Amico JA, Itoua I, Strand HE, Morrison JC et al. (2001). Terrestrial ecoregions of the world: a new map of life on earth. *Bioscience* 51: 933–938.
- Page RDM (1993). *Component 2*. London, UK: The Natural History Museum.
- Pirani JR (1998) A revision of *Helietta* and *Balfourodendron* (Rutaceae-Pteleinae). *Brittonia* 50: 348–380.
- Pombal ECP, Morellato LPC (2000). Differentiation of floral color and odor in two fly pollinated species of *Metrodorea* (Rutaceae) from Brazil. *Plant Syst Evol* 221: 141–156.
- R Development Core Team (2014). R: a language and environment for statistical computing, v. 3. Vienna. Website: <http://www.R-project.org> [accessed 20 March 2014].
- Ronquist F, Huelsenbeck JP (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.
- Santos AMM, Cavalcanti DR, Silva JMC da, Tabarelli M (2007a). Biogeographical relationships among tropical forests in north-eastern Brazil. *J Biogeogr* 34: 437–446.
- Santos K, Kinoshita LS, Santos FA (2007b). Tree species composition and similarity in semideciduous forest fragments of southeastern Brazil. *Biol Conserv* 135: 268–277 (article in Portuguese with an abstract in English).
- Schwarz KD, Pataca CL, Abreu AG, Bariani JM, Macrini CMT, Solferini VN (2010). Genetic diversity in Atlantic forest trees: fragmentation effects on *Astronium graveolens* (Anacardiaceae) and *Metrodorea nigra* (Rutaceae), species with distinct seed dispersal strategies. *Bot J Linn Soc* 164: 326–336.
- Souza LA, Moscheta IS, Mourao KSM, Rosa SM (2004). Morphology and anatomy of the flower and anthesis of *Metrodorea nigra* St. Hill. (Rutaceae). *Braz Arch Biol Techn* 47.1: 107–112.
- Stanford AM, Harden R, Parks CR (2000). Phylogeny and biogeography of *Juglans* (Juglandaceae) based on matK and ITS sequence data. *Amer J Bot* 87: 872–882.
- Swofford DL (2002). PAUP*. *Phylogenetic Analysis Using Parsimony (*and other methods)*, version 4. Sunderland, MA, USA: Sinauer Associates.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 24: 4876–4882.
- Toniato MTZ, Oliveira-Filho AT (2004). Variations in tree community composition and structure in a fragment of tropical semideciduous forest in southeastern Brazil related to different human disturbance histories. *Forest Ecol Manag* 198: 319–339.
- USBGN (1963–onwards). United States Board on Geographic Names gazetteers. Washington, DC: United States National Imagery and Mapping Agency. Website: <http://egsc.usgs.gov/nimamaps/bgn.html> [accessed 6 May 2008].
- Veller MGP van, Brooks DR (2001). When simplicity is not parsimonious: a priori and a posteriori methods in historical biogeography. *J Biogeogr* 28: 1–11.
- Wiley EO (1987). *Methods in vicariance biogeography*. In: Hovenkamp P, editor. *Systematics and evolution: a matter of diversity*. Utrecht, the Netherlands: Utrecht University, pp. 283–306.
- Woodward SL (2009). *Introduction to biomes. Biomes of the World*. Westport, CT, USA: Greenwood Publishing Group.