Polyphyly of *Mussaenda* inferred from ITS and *trnT-F* data and its implication for generic limits in Mussaendeae (Rubiaceae)[1]

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Although recognition of *Mussaenda* as a separate genus has been widely accepted, its generic circumscriptions have always been controversial. In this first molecular phylogenetic study focused specifically on *Mussaenda* sensu lato (s.l.) and its allied genera, parsimony analyses were based on both ITS and *trnT-F* sequence data to (1) test the monophyly of *Mussaenda* s.l. as presently circumscribed, (2) assess the phylogenetic relationships within the tribe Mussaendeae as currently delimited, (3) evaluate the phylogenetic value of the morphological characters traditionally and/or currently used to circumscribe Mussaendeae, (4) and make inferences on the biogeographical origin of *Mussaenda*. Of the 63 *trnT-F* and 38 ITS sequences included in our studies, 52 and 36 sequences, respectively, are newly published here. Our results highly support the polyphyly of *Mussaenda* s.l. as currently delimited but further support the monophyly of Mussaendeae sensu Bremer and Thulin. The Malagasy *Mussaenda* are more closely related to *Landiopsis* than they are to the African and Asian *Mussaenda*. *Pseudomussaenda* and the Afro-Asian *Mussaenda* clade are resolved as sister groups. *Aphaenandra* is nested within the Afro-Asian *Mussaenda* clade. As a result, we merge *Aphaenandra* in *Mussaenda*, which is now restricted to include only the African and Asian *Mussaenda* representatives. We describe a new genus *Bremeria* to accommodate all Indian Ocean (Madagascar and the Mascarenes) *Mussaenda* species and make 19 new combinations. The newly delimited *Mussaenda* is diagnosed by reduplicate-valvate aestivation and glabrous styles, whereas *Bremeria* can be distinguished from the remaining Mussaendeae genera by having both reduplicate- and induplicate-valvate aestivation and densely pubescent styles. Our studies strongly suggest an African origin of the newly delimited *Mussaenda*. Finally, descriptions of the newly circumscribed *Mussaenda* and *Bremeria* are provided.

Key words: biogeography; *Bremeria*; ITS; *Mussaenda*; Mussaendeae; Rubiaceae; *trnT-F*.

Recent phylogenetic analyses within Rubiaceae (or coffee family) based on the *rbcL* sequence data conducted by Bremer and Thulin (1998) led to the reestablishment of the tribe Mussaendeae and proposition of the new tribal circumscriptions for the tribe Isertieae. Mussaendeae, currently belonging to the subfamily Ixoroideae sensu lato (s.l.) (Bremer et al., 1999), comprises seven genera (Bremer and Thulin, 1998): *Aphaenandra* Miq., *Heinsia* DC., *Landiopsis* Capuron ex Bosser, *Mussaenda* s.l. Burm. ex L., *Neomussaenda* Tange, *Pseudomussaenda* Wernham, and *Schizomussaenda* Li. *Mussaenda* s.l. is the most species-rich genus with ca. 163 species of small trees, scandent or scrambling shrubs or true lianas. The genus is mostly paleotropical and has its center of diversity in tropical Asia with ca. 100 species, followed by tropical Africa with ca. 35 species (Bridson and Verdcourt, 1988), Madagascar with ca. 24 species (S. Andriambololonera and S. Razafimandimbison, Missouri Botanical Garden and Bergius Foundation, respectively, unpublished manuscript), and the Mascarenes with four species (Wernham, 1914; Andriambololonera and Razafimandimbison, unpublished manuscript). *Mussaenda* s.l. is characterized by a combination of valvate aestivation, fleshy or berry-like, indehiscent fruits, and numerous, small, reticulate seeds. Many species of *Mussaenda* s.l. (e.g., *M. erythrophylla*, *M. incana*, *M. parvifolia*, *M. philippica*) are commonly cultivated in botanical gardens throughout the world because of their beautiful, long-blooming, sturdy flowers with enlarged calyx lobes.

Although recognition of *Mussaenda* as a separate genus has never been challenged, its circumscription has always been controversial (e.g., Miquel, 1857; Hooker, 1880; Kurz, 1887; Schumann, 1891). Earlier authors disagreed as to whether *Mussaenda* should include only the Asian and African species with enlarged, petaloid calyx lobes or calycophylls (also called semaphylls sensu Leppik, 1977) and fleshy, indehiscent fruits. Miquel (1857) transferred the Asian capsular-fruited *Mussaenda uniflora* without enlarged calyx lobes, described by G. Don (1834), to his new genus *Aphaenandra*. Similarly, both Wernham (1916) and Li (1943) described *Pseudomussaenda* and *Schizomussaenda*, respectively, to accommodate all African and another Asian capsular-fruited *Mussaenda* with enlarged

calyx lobes. On the other hand, Candolle (1830), also endorsed by Hooker (1880), Kurz (1887), and Schumann (1891), recognized a broad circumscription including *Mussaenda* species with or without semaphylls and with dehiscent or indehiscent fruits. This broad circumscription appears never to have gained acceptance. *Mussaenda* s.l. as presently circumscribed (e.g., Robbrecht, 1988; Puff et al., 1993; Mabberley, 1997) includes all Afro-Asian and Malagasy *Mussaenda* species with indehiscent fruits and with or without semaphylls. This situation raises questions as to whether one of these three conflicting generic limits circumscribes a monophyletic unit. The present study is the first phylogenetic investigation to focus specifically on *Mussaenda* s.l. and its alliances.

Previous phylogenetic studies of some Rubiaceae groups have shown that both the internal transcribed spacer (ITS) region of nuclear rDNA (e.g., Andreasen et al., 1999; Persson, 2000; Razafimandimbison and Bremer, 2001) and the *trnT-F* region of chloroplast DNA (e.g., Razafimandimbison and Bremer, 2002) were useful for assessing phylogenetic relationships at both generic and tribal levels. The first objective of the present study is to reconstruct robust phylogenies for *Mussaenda* s.l. and its allied genera using both the ITS and *trnT-F* sequence data. The resulting phylogenies will then be used to: (1) test the monophyly of *Mussaenda* s.l., (2) assess the phylogenetic relationships within the tribe Mussaendeae as currently delimited, (3) evaluate the phylogenetic value of the morphological characters traditionally and/or currently used to circumscribe Mussaendeae, and (4) make inferences on the biogeographical origin of *Mussaenda*.

MATERIALS AND METHODS

Taxon sampling—Material was available for all genera currently placed in Mussaendeae sensu Bremer and Thulin (1998) except for *Neomussaenda*. A total of 37 Mussaendeae species, representing four individuals of *Aphaenandra*, three *Heinsia* species, three *Pseudomussaenda* and 25 *Mussaenda* s.l. species, as well as one individual each of the two monotypic genera *Landiopsis* and *Schizomussaenda*, was included in our analyses. Twelve genera (*Acranthera*, *Gonzalagunia*, *Hippotis*, *Hoffmania*, *Isertia*, *Mycetia*, *Pauridiantha*, *Pentagonia*, *Pseudosabicea*, *Sabicea*, *Schradera*, and *Sommera*) traditionally associated with Mussaendeae and Isertieae sensu Robbrecht (1988) were also added in the *trnT-F* analysis to test the monophyly of Mussaendeae sensu Bremer and Thulin (1998). Few representatives of Cinchonoideae sensu Robbrecht (1988), Ixoroideae, and Rubioideae were additionally investigated (see Appendix in Supplemental Data accompanying online version of this article). The genus *Luculia*, which has been shown to be basal in Rubiaceae (Bremer et al., 1999), was used as the outgroup to root the *trnT-F* tree. Origins and voucher specimens are listed in Appendix.

DNA extraction and amplification—Total DNA was extracted from fresh, silica-gel dried leaf tissues (Chase and Hills, 1991) or herbarium material using DNeasy Plant Mini kit (Qiagen, Hilden, Germany) and cleaned with Qia-Quick PCR purification kit (Qiagen). For amplification and sequencing of the *trnT-F*, the protocols are described in Razafimandimbison and Bremer (2002).

The ITS region (ITS1, 5.8S gene, and ITS2) was amplified using primers P17F (5′-CTA CCG ATT GAA TGG TCC GGT GAA-3′) and 26S–82R (5′-TCC CGG TTC GCT CGC CGT TAC TA-3′) (Popp and Oxelman, 2001). PCR cocktails were mixed as follows (25 μL): 15.3 μL dH$_2$O, 2.5 μL 10× PCR buffer, 2.0 μL 25 mM MgCl$_2$, 1.5 μL 2 mM dNTP, 1.0 μL of 10 μM forward and reverse primers, respectively, 0.2 μL *Taq* DNA polymerase, and 1.5 μL DNA. The Q-solution (Qiagen) was also used as additive replacing some of the water. PCR reactions were run on a Biometra UNO-Thermoblock cycler with initial denaturation for 90 s at 97°C, followed by 35 cycles of 20 s 97°C, 90 s 72°C, 1 : 30 s 72°C, finishing with 72°C for 7 min. PCR products were cleaned with Qia-Quick PCR purification kit (Qiagen).

Sequencing reactions were done using primers P16F (5′-TCA CTG AAC CTT ATC ATT TAG AGG-3′) and P25R (5′- GGG TAG TCC CGC CTG ACC TG-3′) (Popp and Oxelman, 2001) and the ABI PRISM Big Dye Terminator Cycle sequencing kit (Applied Biosystems, Bayreuth, Germany). All sequencing was performed on an ABI Prism Model 310, version 3.0 sequencer.

Data analysis—The ITS and *trnT-F* sequences were assembled using the Perkin Elmer Sequence Navigator, version 1.0.1 and Sequencher 3.1.1, respectively, and edited manually. All new sequences were submitted to EMBL, and their accession numbers are in Appendix (see Supplemental Data for online version of this article). We performed parsimony phylogenetic analyses at two distinct but interrelated levels. We initially conducted a large-scale phylogenetic analysis based on the *trnT-F* data, including the 36 taxa of Mussaendeae sensu Bremer and Thulin (1998), 12 genera previously placed in Mussaendeae, 14 distantly related Rubiaceae taxa from Cinchonoideae, Ixoroideae s.l., and Rubioideae, and one outgroup taxon, for a total of 63 taxa. The results of this analysis allowed us to select new outgroup taxa (*Sabicea diversifolia* and *Warszewiczia coccinea*) from within Ixoroideae s.l. to root both the ITS and combined ITS-*trnT-F* analyses of taxa from Mussaendeae sensu Bremer and Thulin (1998). The parsimony analyses of the ITS, *trnT-F,* and combined ITS-*trnT-F* data sets (excluding uninformative characters) were performed with PAUP* version 4.0b (Swofford, 2000) on a Power Macintosh G3 computer using heuristic searches, with the MULTREES option on, tree-bisection-reconnection (TBR) branch swapping, swap on best only in effect, and 5000 random addition sequences. The heuristic search for the *trnT-F* analysis could not be completed due to computational limitations. The *trnT-F* data were then analyzed using the following settings: the MULTREES option off, nearest neighbor interchanges (NNI) branch swapping, and 10 000 random addition sequences. For the combined ITS-*trnT-F* data sets, we likewise searched for multiple islands of most-parsimonious trees (Maddison, 1991). In all analyses, characters were given equal weight, gaps were treated as missing data, and phylogenetically informative indels were coded following the simple gap coding method of Simmons and Ochoterena (2000). The consistency index (CI; Kluge and Farris, 1969) and retention index (RI; Farris, 1989) were calculated to estimate homoplasy. Bootstrap (BS; Felsenstein, 1985) values using 10 000 replicates, the MULTREES option off, NNI branch swapping, and five random addition sequences were performed to assess relative support for the identified clades. Clades receiving a bootstrap support of 50–69% were regarded as weakly supported, 70–85% as moderately supported, and 86–100% as strongly supported.

We statistically evaluated the combinability of the ITS and *trnT-F* data partitions using the one-tailed Shimodaira-Hasegawa test (SH test; Shimodaira and Hasegawa, 1999; Goldman et al., 2000) and the incongruence length difference (ILD test; Farris et al., 1995), both implemented in PAUP*. We perfomed maximum likelihood (ML) analyses of the ITS and *trnT-F* data, respectively, using the GTR + G + I and the GTR + G substitution models, which were selected by MrModeltest (Nylander, 2002) as the best models. We subsequently conducted the SH tests, using resampling estimated by log-likelihood (RELL) optimization and 1000 bootstrap replicates, to compare statistically the optimal ITS and *trnT-F* topologies, respectively, against two alternative phylogenetic hypotheses: topology inferred from the ITS data constrained by the optimal ML topology of the *trnT-F* data (for the ITS matrix) and topology from the *trnT-F* data constrained by the best topology from the ITS data (for the *trnT-F* partition).

Incongruency test was performed using the incongruence length difference (ILD test; Farris et al., 1995) to assess incongruencies between the ITS and *trnT-F* data sets. This test uses the partition-homogeneity test as implemented in PAUP* (Swofford, 2000). The heuristic search was set to 500 replicates with 10 random addition sequence and NNI branch swapping. If the probability of obtaining a smaller sum of tree lengths from the randomly generated data sets is lower ($P \leq 0.05$) than that of the original data sets, the null hypothesis that the two data sets are homogenous is rejected and they are interpreted as incongruent (Farris et al., 1995).

Finally, additional SH tests were performed to test whether or not the optimal ML topologies of both the ITS (Fig. 1) and *trnT-F* (Fig. 2) trees were significantly different from the alternative hypothesis constraining all sampled African *Mussaenda* species monophyletic.

RESULTS

Sequence characteristics—The *trnT-F* sequences of the sampled members of Mussaendeae varied from 1703 base pairs (bp) (*Heinsia crinita*) to 1793 bp (*Mussaenda latisepala*). The total GC content of the *trnT-F* Mussaendeae sequences ranged from 30.82% (*Pseudomussaenda flava*) to 36.64% (*Mussaenda isertiana*) and its average was 31.55%. The ITS sequences of the sampled members of Mussaendeae varied from 570 bp (all Malagasy *Mussaenda* included) to 596 bp (*Heinsia bussei* and *H. zanzibarica*). The average total length of ITS1 and ITS2 were 204 and 221 bp, respectively. From all Mussaendeae sequences included, ITS2 (215–223 bp) was longer than ITS1 (185–209 bp), consistent with the earlier report in *Mussaenda erythrophylla* (Andreasen et al., 1999). The average length falls within the range for other angiosperms (ITS1: 187–298 bp and ITS2: 187–252 bp; Baldwin et al., 1995). The total GC content of the entire ITS region ranged from 59.80% (*Schizomussaenda dehiscens*) to 63.76% (*Landiopsis capuronii*) and its average was 61.19%.

TrnT-F analysis—Of 63 *trnT-F* sequences included in our studies, 52 are newly published here. The non-aligned *trnT-F* sequences ranged from 1662 bp (*Gonzalagunia affinis*) to 1793 bp (*Mussaenda latisepala*). The *trnT-F* alignment of 63 taxa consisted of 2263 positions, 45 (1.99%) of which were coded as phylogenetically informative indels and 508 (22.45%) were phylogenetically informative characters. Of these informative characters, 314 (61.81%) were from the *trnT-L* spacer, 81 (15.94%) from the *trnL* intron, and 113 (22.24%) from the *trnL-F* spacer. Within Mussaendeae, alignment of 37 taxa consisted of 1924 positions and contained 131 (7.31%) phylogenetically informative characters. Parsimony analyses of the 63 *trnT-F* sequences data resulted in 1410 equally parsimonious trees (each 1207 steps long [L], CI = 0.638, and RI = 0.834). In the strict consensus tree shown in Fig. 1, all investigated members of Mussaendeae sensu Bremer and Thulin (1998) formed a strongly supported (BS = 100) monophyletic group. Within the Mussaendeae clade, a total of four major clades were resolved: (1) a strongly supported (BS = 100) clade containing all sampled *Heinsia* species; (2) a highly supported (BS = 100) monophyletic group comprising the sampled *Pseudomussaenda* species; (3) a moderately supported (BS = 80) clade forming three African *Mussaenda* species (*M. afzelii*, *M. grandiflora*, and *M. isertiana*); and (4) a weakly supported (BS = 63) clade containing all investigated Asian *Mussaenda*, *Aphaenandra uniflora*, and five African *Mussaenda* species (*M. arcuata*, *M. elegans*, *M. erythrophylla*, *M. monticola*, and *M. nivea*). All sampled Malagasy *Mussaenda*, *Landiopsis capuronii*, and *Schizomussaenda dehiscens* were left unresolved. *Mussaenda* s.l. as presently delimited was shown to be polyphyletic because the Afro-Asian *Mussaenda* species were not directly related to the Malagasy *Mussaenda* species. Plus, the *Pseudomussaenda* clade was resolved with high support (BS = 90) as sister to the Afro-Asian *Mussaenda* clade. Furthermore, all sampled individuals of *A. uniflora* formed a strongly supported (BS = 99) monophyletic group, which was embedded within the Afro-Asian *Mussaenda* clade. Similarly, we

perceived no support for the monophyly of the narrow circumscription of *Mussaenda* that included only the Afro-Asian *Mussaenda* with semaphylls and dehiscent fruits (e.g., Miquel, 1857; Wernham, 1916). In contrast, the broadly circumscribed *Mussaenda* including all species with and without semaphylls and with dehiscent or indehiscent fruits (e.g., Candolle, 1830; Hooker, 1880; Kurz, 1887; Schumann, 1891) was resolved with high support (BS = 98) as monophyletic. This clade was resolved with strong support (BS = 100) as sister to the *Heinsia* clade.

Mussaendeae sensu Bremer and Thulin (1998) was resolved with strong support (BS = 94) as sister to Sabiceeae (represented by *Sabicea diversifolia* and *Pseudosabicea becquetii*) and placed within Ixoroideae s.l. The remaining Mussaendeae-associated genera included in our study were resolved with high support in the three subfamilies: both *Mycetia* and *Schradera* in Rubioideae sensu Bremer and Manen (2000); *Hoffmania*, *Gonzalagunia*, and *Isertia* all in Cinchonoideae sensu stricto (s.s.) (Bremer et al., 1995; Bremer et al., 1999); and *Hippotis*, *Pentagonia*, and *Sommera* all in Ixoroideae s.l. (Bremer et al., 1999; Rova et al., 2002). Finally, *Acranthera* and *Mussaendopsis* were placed with strong support in Rubioideae and Ixoroideae s.l., respectively.

ITS analysis—A total of 38 ITS sequences were included and 36 are newly published here. The aligned matrix contained 655 positions and 103 (15.72%) were phylogenetically informative, eight (1.22%) of which were coded as phylogenetically informative indels. Of these informative characters, 48 (46.60%) were from the ITS1, 53 (51.46%) from the ITS2, and only two (1.94%) from the 5.8S gene. A parsimony analysis of the ITS data resulted in 524 equally parsimonious trees (L = 279, CI = 0.599, and RI = 0.811). In the strict consensus tree shown in Fig. 2, all investigated members of Mussaendeae sensu Bremer and Thulin (1998) resolved four major clades: (1) the *Heinsia* clade (BS = 100); (2) a strongly supported (BS = 100) clade containing *Landiopsis* and the sampled Malagasy *Mussaenda* species; (3) the *Pseudomussaenda* clade (BS = 98); and (4) a moderately supported (BS = 72) clade containing *Aphaenandra uniflora* and all sampled African and Asian *Mussaenda*. Similar with the *trnT-F* tree (Fig. 1), the *Heinsia* clade was resolved as a sister to a clade containing the other sampled members of Mussaendeae. The *Landiopsis*-Malagasy *Mussaenda* clade was resolved with high support (BS = 90) as sister to a moderately supported clade forming all investigated *Schizomussaenda*, *Pseudomussaenda*, and all Afro-Asian *Mussaenda* species. Finally, all sampled individuals of *A. uniflora* and three African *Mussaenda* (*M. afzelii*, *M. grandiflora*, and *M. isertiana*) constituted strongly supported (BS = 99 and 100, respectively) monophyletic groups.

Combined analysis—The results of the SH and partition-homogeneity tests (Tables 1, 2, respectively) both showed that the ITS and *trnT-F* data sets were significantly incongruent. Visual inspection of the *trnT-F* and ITS trees (Figs. 1, 2) revealed topological conflicts regarding the placement of the sampled Asian *Mussaenda* and *Schizomussaenda dehiscens*. The sampled Asian *Mussaenda* together with five African *Mussaenda* (*M. arcuata*, *M. elegans*, *M. erythrophylla*, *M. monticola*, and *M. nivea*) were resolved with weak support (BS = 63) as a monophyletic group in the *trnT-F* tree. In contrast, these Asian *Mussaenda* species together with three African
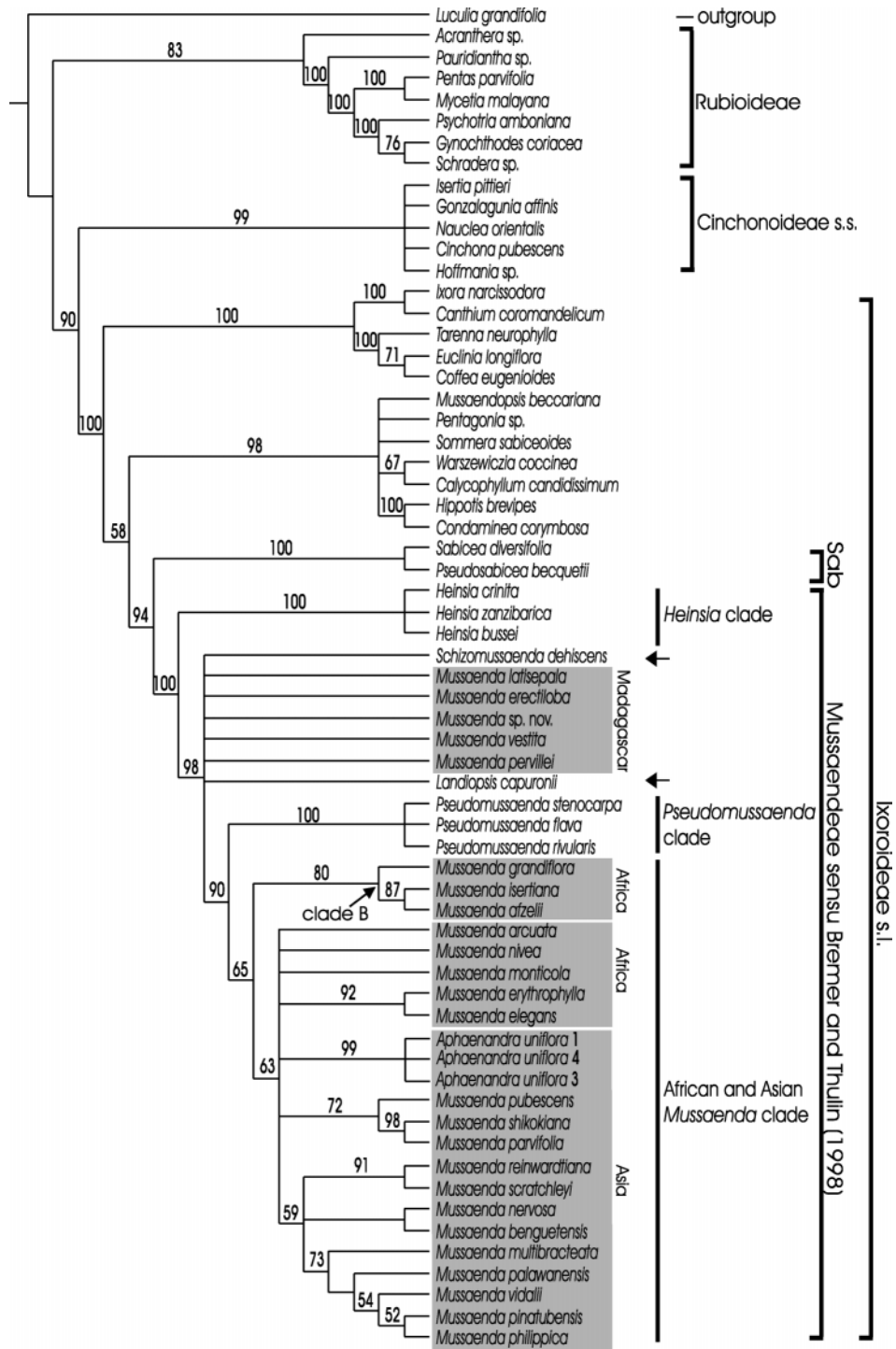
Fig. 1. Strict consensus tree derived from 1410 equally parsimonious trees based on the phylogenetic analysis of *trnT-F* sequence data. Numbers above nodes are bootstrap support values >50%. The thin horizontal bar at the top corresponds to the outgroup, and thick bars indicate clades resolved within Mussaendeae. Arrows indicate the positions of *Landiopsis capuronii* and *Schizomussaenda dehiscens*. Brackets indicate tribal limits of Sabiceeae (Sab) and Mussaendeae and subfamilial limits.

Mussaenda (*M. afzelii*, *M. grandiflora*, and *M. isertiana*) formed a strongly supported (BS = 90) clade in the ITS tree. In the ITS tree (Fig. 2), *S. dehiscens* left unresolved within a poorly supported (BS = 64) clade that also contains the *Pseudomussaenda* and the Afro-Asian *Mussaenda* subclades. In contrast, this species was left unresolved outside the *Pseudo-*

mussaenda-Afro-Asian *Mussaenda* clade in the *trnT-F* tree (Fig. 1). The two data sets became significantly congruent (*P* = 0.294, Table 1) when *S. dehiscens* and all sampled Asian *Mussaenda* species were excluded. When we excluded *S. de-hiscens* and restored all sampled Asian *Mussaenda* species, the two data sets were still significantly congruent (*P* = 0.140,
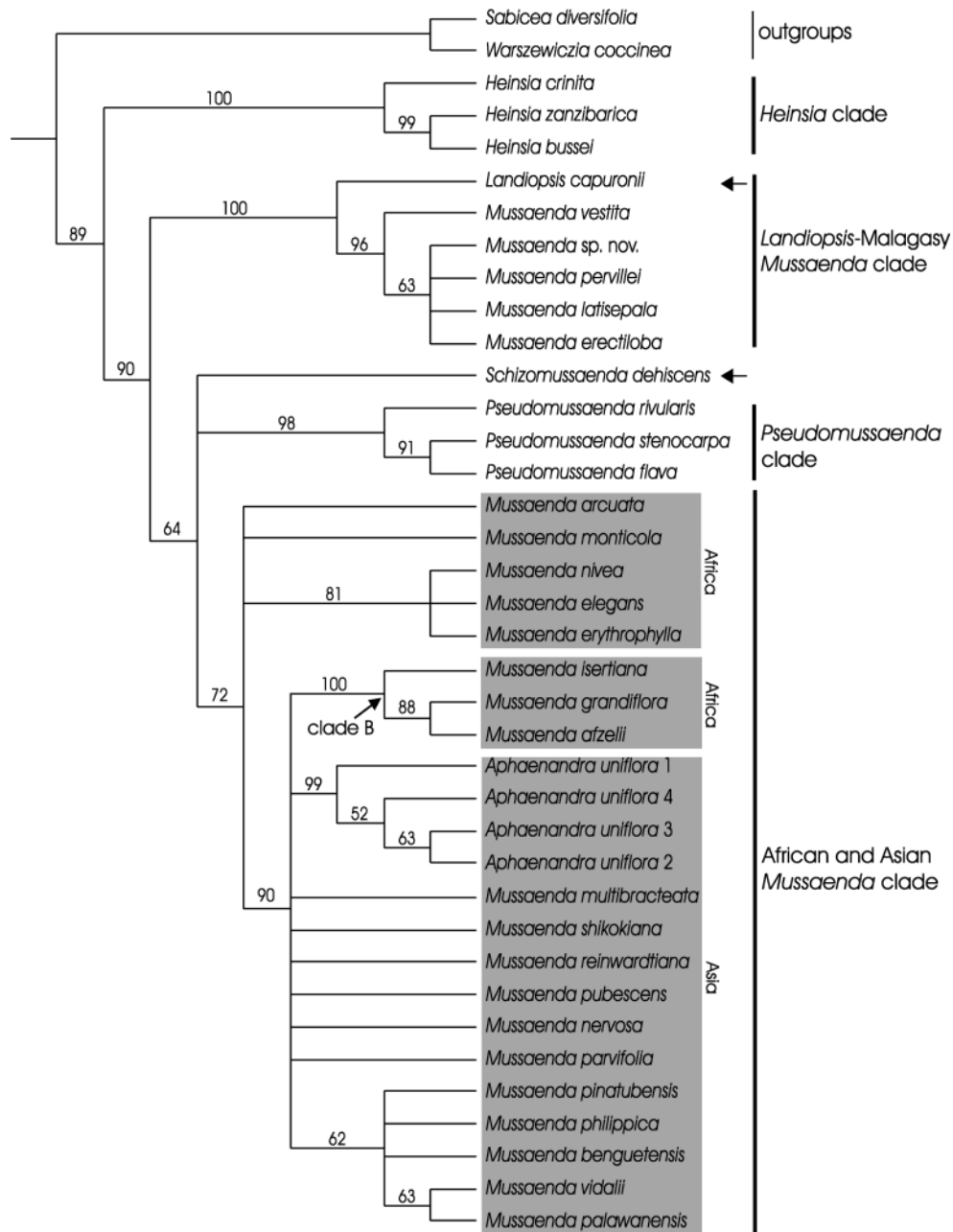
Fig. 2.   Strict consensus tree derived from 524 equally parsimonious trees based on the phylogenetic analysis of ITS sequence data. Numbers above nodes are bootstrap support values >50%. The vertical thin bar indicates the outgroups, and thick bars indicate clades resolved within Mussaendeae. Arrows indicate the positions of *Landiopsis capuronii* and *Schizomussaenda dehiscens*.

TABLE 1.   Log likelihood scores of ITS and *trnT-F* partitions combinability implementing the Shimodaira-Hasegawa (SH) test (*$P < 0.05$).

Partition	Constraint	Score (−lnL)	Difference (−lnL)	Significance (P)
ITS	Optimal ML topology	2664.339 64	best	
	constrained by *trnT-F* topology	2730.967 74	66.628 10	0.000*
trnT-F	Optimal ML topology	4669.527 04	best	
	constrained by ITS topology	4759.832 01	90.304 97	0.004*

TABLE 2.   Results from the incongruence length difference (ILD) test.

Taxa included	P values
All sampled Mussaendeae taxa + two outgroups (*Sabicea diversifolia* and *Warszewiczia coccinea*)	0.018
All *Schizomussaenda dehiscens* + Asian *Mussaenda* species + *Aphaenandra uniflora* excluded	0.294
Only *Schizomussaenda dehiscens* excluded	0.140
All sampled Asian *Mussaenda* species + *Aphaenandra uniflora* excluded	0.032

Table 1). In contrast, when we excluded all sampled Asian *Mussaenda* species and restored *S. dehiscens*, they became significantly incongruent ($P = 0.032$, Table 1). Based on this evidence we combined the two data sets (excluding *S. dehiscens*) in one large matrix, which comprised 2579 bp (including coded indels); 229 (8.87%) of these 2579 bp were parsimony-informative characters. Parsimony analyses of the combined ITS-*trnT-F* of 36 taxa resulted in three islands containing 240 most equally parsimonious trees ($L = 462$, $CI = 0.660$, and $RI = 0.840$). The strict consensus tree shown in Fig. 3 was almost fully resolved and retained almost the same large monophyletic groups found in both the *trnT-F* and ITS trees (Figs. 1, 2). The sampled African *Mussaenda* were resolved in two separate clades: the weakly supported ($BS = 64$) African clade A (containing *M. arcuata*, *M. elegans*, *M. erythrophylla*, *M. monticola*, and *M. nivea*); and the strongly supported ($BS = 100$) African clade B (forming *M. afzelii*, *M. grandiflora*, and *M. isertiana*), which was resolved with high support ($BS = 89$) as sister to all the investigated Asian *Mussaenda* species. The African *Mussaenda* clade A collapsed in both the *trnT-F* and ITS trees (Figs. 1, 2).

Furthermore, the results of SH tests additionally showed that the optimal ML topologies of both the ITS (Fig. 1) and *trnT-F* (Fig. 2) trees were not significantly different from the alternative hypothesis constraining all sampled African *Mussaenda* species monophyletic (Table 3).

DISCUSSION

Data sets comparison within Mussaendeae—Although the *trnT-F* region is three times longer than the ITS region, the latter yields more informative characters (15.72%) than the former (7.31%), consistent with the conclusions of Razafimandimbison and Bremer (2002) on Naucleeae s.l. Our results additionally show that the *trnT-L* spacer (with 102 variable sites) and the *trnL-F* spacer (with 37 variable sites) are more variable than the *trnL* intron (with 32 variable sites), also consistent with Razafimandimbison and Bremer (2002). The *trnT-L* spacer also has more phylogenetically informative characters (64.88%) than the *trnL-F* spacer (27.43%), further suggesting that these three regions evolving at different rates are useful for inferring phylogenetic relationships at different taxonomical levels of Rubiaceae (see also Meve and Liede [2002, 2004] for Apocynaceae, Gentianales).

Causes of incongruence between the ITS and trnT-F trees within Mussaendeae sensu Bremer and Thulin (1998)—The results of the partition-homogeneity tests show that *Schizomussaenda dehiscens* causes the significant difference between the *trnT-F* and ITS data sets (Table 2) despite its unresolved positions in both the *trnT-F* and ITS trees (Figs. 1, 2). The results of both the SH (Table 1) and ILD (Table 2) tests seem to indicate that the incongruence regarding the placement of the sampled Asian *Mussaenda* species in the *trnT-F* and ITS

data sets is simply due to lack of enough resolution within the two data sets.

Monophyly of Mussaendeae sensu Bremer and Thulin (1998)—Our results strongly support ($BS = 100$, Figs. 1, 3; $BS = 89$, Fig. 2) the monophyly of Mussaendeae sensu Bremer and Thulin (1998). Although we have not been able to find any morphological synapomorphy to diagnose the tribe, the combination of the following morphological characters commonly found in Mussaendeae can be used to characterize it: bifid stipules, shaggy trichomes, terminal inflorescences, heterostyly, semaphylls, corolla lobes with tail-like projections, discoid placentae, and fruits with tanniniferous idioblasts (Bremer and Thulin, 1998). The *trnT-F* tree (Fig. 1) further corroborates the placement of Mussaendeae in Ixoroideae, also in agreement with Bremer and Thulin (1998) and Rova et al. (2002).

Placements of some traditionally Mussaendeae-associated genera in Rubiaceae—The placement of *Isertia*, *Gonzalagunia*, and *Hoffmania* in Cinchonoideae s.s. (Bremer and Thulin, 1998) is further corroborated by our *trnT-F* tree (Fig. 1). Similarly, the position of *Pauridiantha*, *Mycetia*, and *Schradera* in Rubioideae (e.g., Bremer and Thulin, 1998; Andersson and Rova, 1999; Bremer and Manen, 2000; Rova et al., 2002) are also supported by our results. Four Mussaendeae-associated genera (*Ecpoma*, *Pentaloncha*, *Stipularia*, and *Temnopteryx*), which are not included in the present study due to lack of material, are tentatively placed by Andersson (1996) in Sabiceeae based on morphology. Recently, the placement of *Sommera* and *Pentagonia* (traditionally considered of Cinchonoideae affinity) in Ixoroideae s.l. (Bremer et al., 1999; Rova et al., 2002) is further corroborated by our *trnT-F* tree (Fig. 1).

The position of *Acranthera* within Rubiaceae has always been controversial since its original description. *Acranthera* was originally described by Arnott, but it was Meisner (1838) who validly published it in his survey of Rubiaceae. The genus was traditionally placed in Mussaendeae (e.g., Meisner, 1838; Hooker, 1873; Baillon, 1880; and Schumann, 1891) of Cinchonoideae because of its terminal inflorescences, valvate corolla aestivation, pluriovular-bicarpellate ovaries, and fleshy, indehiscent fruits, features found in *Mussaenda*. However, *Acranthera* always has simple and entire stipules, homostylous flowers, corolla completely glabrous inside, stamens inserted at the base of corolla tube, anthers forming a sheath around the style, and the secondary pollen presentation (Bremekamp, 1947). We agree with Bremekamp (1947) that placing *Acranthera* in Mussaendeae with bifid stipules, heterodistylous flowers, and anthers attached at least inside of densely pubescent corolla tubes would make this tribe rather heterogeneous, morphologically. As a result, Bremekamp (1966) removed *Acranthera* from Mussaendeae and placed it in its own tribe Acranthereae Bremekamp ex Darwin within Ixoroideae. Our
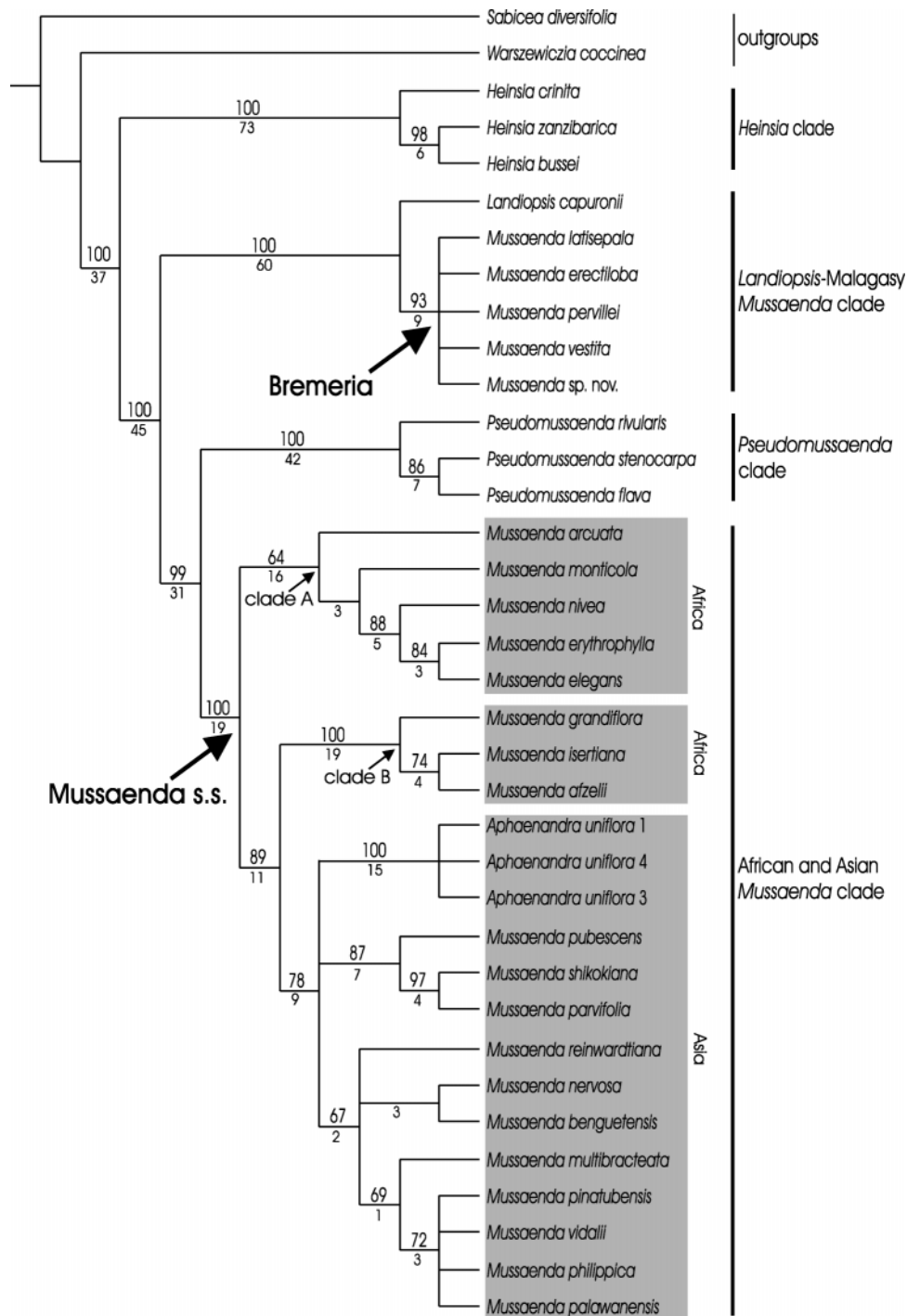
Fig. 3. Strict consensus tree derived from 240 equally parsimonious trees based on the phylogenetic analysis of combined ITS and *trnT-F* sequence data. Numbers above nodes are bootstrap values >50% and below nodes are branch lengths. Vertical thin bar indicates outgroups, and thick bars indicate clades resolved within Mussaendeae.

TABLE 3. Log likelihood scores for two alternative tree topologies using Shimodaira-Hasegawa test ($P < 0.05$).

Partition	Constraint	Score (−lnL)	Difference (−lnL)	Significance (P)
ITS	Optimal ML topology	2664.339 64	best	
	African *Mussaenda* (clades A and B) monophyletic	2677.728 26	13.388 62	0.092
trnT-F	Optimal ML topology	4669.527 04	best	
	African *Mussaenda* (clades A and B) monophyletic	4674.911 43	5.384 40	0.370

trnT-F tree (Fig. 1) places *Acranthera* as sister to the other Rubioideae taxa included in this study.

Razafimandimbison and Bremer's (2001) study based on the *rbcL* sequence data placed *Mussaendopsis* in Ixoroideae s.l., consistent with our present findings. Our study strongly indicates that *Mussaendopsis* belongs to the strongly supported clade containing *Pentagonia*, *Hippotis*, *Condaminea*, *Sommera*, *Warszewicsia*, and *Calycophyllum*. *Mussaendopsis* has enlarged calyx lobes similar to those found in all investigated genera in Mussaendeae as defined here. However, the genus can easily be recognized by its intrapetiolar stipules (Puff and Igersheim, 1994), and it is so far the only Asian member of the clade. This clade was also previously identified by Bremer (1996) and Rova et al. (2002) and is morphologically distinct from the remaining tribes of Ixoroideae s.l.

Polyphyly of Mussaenda s.l.—The analyses presented (Figs. 1–3) all support the monophyly of the broadly circumscribed *Mussaenda* that includes *Aphaenandra*, *Landiopsis*, *Pseudomussaenda*, and *Schizomussaenda*. This circumscription maximizes nomenclatural stability because *Aphaenandra*, *Pseudomussaenda*, and *Schizomussaenda* were originally described as *Mussaenda* species. However, it makes *Mussaenda* highly heterogeneous, morphologically (e.g., with four types of corolla aestivations: imbricate [*Landiopsis*], induplicate-valvate [*Neomussaenda*, *Pseudomussaenda*, and *Schizomussaenda*], reduplicate-valvate [Afro-Asian *Mussaenda*], and induplicate-reduplicate-valvate [Malagasy and Mascarene *Mussaenda*]; with dehiscent and indehiscent fruits).

Our analyses strongly support the polyphyly of *Mussaenda* s.l. as presently delimited. A constrained parsimony analysis of the combined data sets forcing Malagasy *Mussaenda* to be monophyletic with African and Asian *Mussaenda* results in 60 equally most parsimonious trees, each 501 steps long. These trees are 39 steps longer than the trees generated from the unconstrained analyses and therefore are not the most parsimonious solution. Our results indicate that *Mussaenda* s.l. needs to be recircumscribed. Here, we restrict *Mussaenda* to include only the Afro-Asian *Mussaenda* and *Aphaenandra* and recognize the Malagasy and Mascarene *Mussaenda* at generic level. This scenario is consistent with the arguments put forward by Wernham (1914) and Bremekamp (1937) that the Malagasy and Mascarene *Mussaenda* are distinct from the African and Asian *Mussaenda* species because of lack of enlarged calyx lobes and their relatively large flowers. It makes both the Afro-Asian *Mussaenda* and the Malagasy *Mussaenda* clades homogeneous, morphologically, and also reflects the distinctness of these two groups, as well as *Landiopsis*, *Pseudomussaenda*, and *Schizomussaenda*. Furthermore, this involves some nomenclatural changes only for the Indian Ocean (the Malagasy and Mascarene) *Mussaenda* species. The Malagasy *Mussaenda* clade is diagnosed by two morphological synapomorphies: reduplicate- (each lobe folded inward and its entire inner surface in contact with its adjacent lobes; Robbrecht, 1988: 84) and induplicate- (each lobe folded inward and its entire inner surface in contact with its adjacent lobes, Robbrecht, 1988: 84) valvate aestivation (Fig. 4) and densely pubescent styles. Accordingly, we describe a new genus *Bremeria* to accommodate all Malagasy and Mascarene *Mussaenda* species. This generic name honors Professor Birgitta Bremer, who has dedicated her life to the study of Rubiaceae and whose contributions have changed the views of the classifications of this large family. Furthermore, the circumscription

of *Mussaenda* by Miquel (1857), also endorsed by Wernham (1916) and Bremekamp (1937) that restricted *Mussaenda* to the Afro-Asian *Mussaenda* with semaphylls and fleshy indehiscent fruits is not supported by our results, as the two African and Asian *Mussaenda* species (*M. arcuata* and *M. pubescens*, respectively) without semaphylls are both grouped together with the other sampled Afro-Asian *Mussaenda* species with semaphylls. The Afro-Asian *Mussaenda* clade received high support (BS = 100) in our combined tree (Fig. 3), and its members can be diagnosed by their reduplicate-valvate aestivation and ovary walls with laticiferous cells. We propose here a much narrower circumscription of *Mussaenda*, which includes all Afro-Asian species only.

Phylogenetic relationships and generic limits within Mussaendeae sensu Bremer and Thulin (1998)—*Heinsia clade*—Our combined tree (Fig. 3) provides strong support (BS = 100) for the monophyly of *Heinsia*, represented here by three species (*H. bussei*, *H. crinita*, and *H. zanzibarica*). Recognition of *Heinsia* at the generic level has been widely accepted. The genus can easily be recognized by a combination of deeply bifid stipules, imbricate corolla aestivation, fleshy and indehiscent fruits, and numerous exotesta cells mostly with well-protruding tuberculate thickenings along both the radial and inner tangential walls. Accordingly, its current generic status should be retained. The morphology-based phylogeny by Andersson (1996) resolves *Heinsia* as sister to *Aphaenandra*, a relationship not supported by our results, which place *Heinsia* as sister to a clade formed by all sampled members of Mussaendeae.

Landiopsis-Malagasy Mussaenda (Bremeria) clade—Our ITS (Fig. 2) and combined tree (Fig. 3) strongly support (BS = 100) the monophyly of the *Bremeria-Landiopsis* group, which is characterized by having much larger corollas compared to its sister-group (the *Pseudomussaenda*-Afro-Asian *Mussaenda* clade) with smaller corollas. *Landiopsis* (Bosser and Lobreau-Callen, 1998) and *Bremeria* are resolved with strong support (BS = 100) as sister genera in both the ITS (Fig. 2) and combined (Fig. 3) trees, but this relationship collapsed in the *trnT-F* tree (Fig. 1). *Landiopsis* can easily be recognized by its subsessile inflorescences, imbricate aestivation, lenticellate and dehiscent fruits, and nonperforate exine. We have not found any morphological synapomorphy for *Landiopsis* and *Bremeria*. However, the former is restricted to dry habitats in northern Madagascar, whereas the latter is confined to the low and mid-altitude Malagasy and Mascarene rainforests. Therefore, we maintain the current generic status of *Landiopsis*.

Palynological studies of Bosser and Lobreau-Callen (1998) showed evident affinities of *Landiopsis* to *Mussaenda* s.l. and its alliances in having the same apertural system and microendosculptured nexine. As a result, *Landiopsis* was placed in Isertieae sensu Andersson (1996). Our molecular results strongly support the placement of *Landiopsis* in Mussaendeae sensu Bremer and Thulin (1998).

Pseudomussaenda clade—*Pseudomussaenda* was originally described by Wernham (1916) to accommodate all African *Mussaenda* species with dry, capsular fruits and induplicate-valvate aestivation. Our analyses all perceive strong support for the monophyly of *Pseudomussaenda*. Wernham (1916), also endorsed by Robbrecht (1988), tentatively placed *Pseu-*
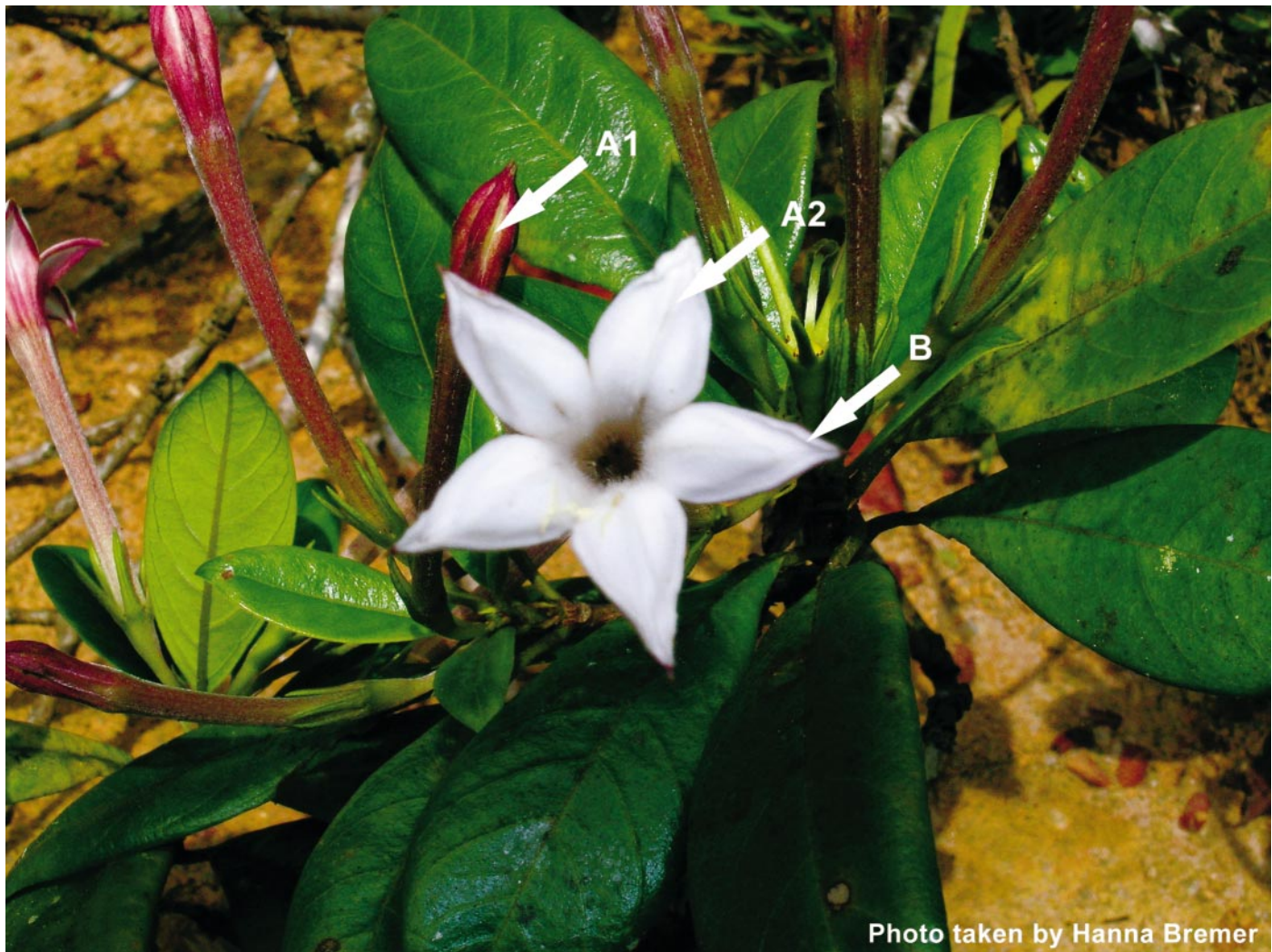
Fig. 4. Flower buds and an opened flower of *Bremeria hymenopogoides* showing induplicate- and reduplicate-valvate aestivation. A1, a distinct median ridge on one of the corolla lobes of a flower bud; A2, a median ridge on one of the corolla lobes of the opened flower (reduplicate-valvate aestivation); B, infolded part of the margin of one corolla lobe of the opened flower (induplicate-valvate aestivation).

domussaenda in the tribe Condamineeae (Cinchonoideae sensu Robbrecht, 1988) because of its capsular fruits. Based on their detailed morphological investigations on some African and Asian *Mussaenda* Puff et al. (1993), however, concluded that *Pseudomussaenda* belongs to Isertieae sensu Robbrecht (1988) and it is more closely related to *Mussaenda* s.l. than it is to the rest of Isertieae. They further argued that this close relationship is not sufficient to warrant their unification. Our combined tree (Fig. 3) resolves with high support (BS = 99) the *Pseudomussaenda* clade as sister to the Afro-Asian *Mussaenda* clade. This is inconsistent with the conclusions of Wernham (1916) and Robbrecht (1988) but consistent with Puff et al. (1993). We have not found any morphological synapomorphy for the *Pseudomussaenda* and Afro-Asian *Mussaenda* clade.

It is worth noting that our results appear to conflict with the conclusions of Puff et al. (1993) because their generic circumscription of *Mussaenda* s.l. includes the Indian Ocean (Madagascar and Mascarene) *Mussaenda* species; such circumscription is strongly supported to be polyphyletic by our studies. However, their studies were based only on some Afro-Asian *Mussaenda*; therefore, our findings are actually consistent with

their conclusions. Puff et al. (1993) additionally show that the ovary walls of the Afro-Asian *Mussaenda* always contain laticiferous cells, which are absent in *Pseudomussaenda*. Bridson and Verdcourt (1988) argue that *Pseudomussaenda* can be distinguished from *Mussaenda* s.l. by having five filiform corolla lobe appendages. However, Puff et al. (1993) show that the filiform appendages are also present in some *Mussaenda* species. Plus, these features are also found in many Philippian *Mussaenda* species (Alejandro, personal observation). Like *Schizomussaenda*, *Pseudomussaenda* has valvate-induplicate corolla aestivation and dry, capsular fruits. *Schizomussaenda* are with long corollas (always >5 cm) and the inner walls of seeds with small pits; *Pseudomussaenda*, however, are with shorter corollas (always <5 cm) and the inner walls of seeds with conspicuous large pits (Puff et al., 1993). Plus, *Pseudomussaenda* is restricted to mainland Africa, whereas *Schizomussaenda* is exclusively Southeast Asian. Accordingly, we maintain the current generic status of *Pseudomussaenda*.

Afro-Asian Mussaenda clade—The Afro-Asian *Mussaenda* clade corresponds to our newly circumscribed *Mussaenda* s.s.

TABLE 4.   List of genera accepted here and their synonyms, geographic distributions, and number of species.

Accepted genera in Mussaendeae	Synonyms	Geographic distributions	Number of species
Bremeria Razafim. and Alejandro		Madagascar and the Mascarenes	28
Heinsia DC.		Mainland Africa	4–5*
Landiopsis Capuron ex Bosser		northern Madagascar	monotypic
Mussaenda s.s. Burm. ex L.	*Aphaenandra* Miq.; *Landia* Comm. ex Juss.	Mainland Africa and Asia	132
Neomussaenda Tange		Southeast Asia	2
Pseudomussaenda Wernham		Mainland Africa	4–5*
Schizomussaenda Li		southwestern China westwards to northern Myanmar	monotypic

 * Number of species taken from Mabberley (1997).

The morphological tree shown in Andersson (1996, p. 154) resolves with strong support the Afro-Asian *Mussaenda* clade, represented by three African *Mussaenda*, *M. arcuata*, *M. glabra*, and *M. pubescens*, as sister to a clade formed by *Pseudomussaenda* and *Schizomussaenda*. Our combined tree (Fig. 3), however, resolves with strong support (BS = 99) the Afro-Asian *Mussaenda* clade as sister to *Pseudomussaenda*, consistent with the conclusions of Puff et al. (1993).

The Southeast Asian genus *Aphaenandra* is nested with the Afro-Asian *Mussaenda* clade (Figs. 1–3). Miquel (1857) originally described *Aphaenandra* based on *A. sumatrana*, which he tentatively placed in Rondeletieae. Since then, both its identity and position within Rubiaceae have always been under debate. Hooker (1873), also endorsed by Schumann (1897), considered *Aphaenandra* as a dubious genus because of its suffrutescent habit and mode of vegetative propagation via stolons, making it rather unique within Rubiaceae. De Voogd (1929), endorsed by Jochems (1929), Craib (1932), Bremekamp (1937), and Robbrecht (1988), all emphasized the striking similarities between *Aphaenandra* and *Mussaenda* s.l.: bifid stipules, heterodistylous but functionally dioecious flowers, upper half of the inside of corolla tubes covered with yellow hairs, stamens inserted in or above the middle, styles with two filiform stigmas, and peltate placentae. Craib (1932) reduced *A. sumatrana* under synonymy of *Mussaenda uniflora* Wall. ex G. Don. Bremekamp (1937), however, argued that both the small suffrutescent habit and the vegetative propagation mode of *Aphaenandra* are sufficient for retaining it as a separate genus. Accordingly, he resurrected *Aphaenandra* from synonymy and subsequently made the new combination of *Aphaenandra uniflora* (Wall. ex G. Don) Bremekamp. The morphologically based phylogenetic study by Andersson (1996, p. 154) resolves *Aphaenandra* as sister to *Heinsia*. This sister-genera relationship is not supported by our results (Figs. 1–3) because all sampled individuals of *Aphaenandra uniflora* form a strongly supported monophyletic group, which is always embedded within the Afro-Asian *Mussaenda* clade. Therefore, our findings are consistent with Craib's decision but inconsistent with the conclusions of Bremekamp (1937). This placement of *Aphaenandra* is further supported by morphological data, because it also has typical reduplicate-valvate aestivation, the same basic chromosome number ($x = 11$), and ploidy level (diploid) of the Afro-Asian *Mussaenda* (Puangsomlee and Puff, 2001). All of the calyx lobes of *Aphaenandra* are subequal, a feature also found in some Afro-Asian *Mussaenda* species (e.g., *M. arcuata* and *M. elegans*). Furthermore, the same functionally dioecious flowers have also been discovered in the Japanese *Mussaenda parviflora* (Naiki and Kato, 1999) and most Philippian *Mussaenda* species (Alejan-

dro, personal observation). So, merging *Aphaenandra* in *Mussaenda* is not anomalous as Bremekamp (1937) claimed. Based on all our evidence presented, we sink *Aphaenandra* in the newly circumscribed *Mussaenda* s.s. Jochems (1929) pointed out that the fruits of *Aphaenandra* had a dehiscent opening in the end, splitting the fruit in two halves. We investigated about 40 specimens of *Aphaenandra uniflora* and did not find any dehiscent, capsular fruits; all mature fruits appear to be fleshy and indehiscent.

Schizomussaenda—*Schizomussaenda dehiscens* (Li, 1943) has the same type of induplicate-valvate aestivation as that found in both *Neomussaenda* and *Pseudomussaenda* and dry, capsular fruits, which are also characteristics for both *Landiopsis* and *Pseudomussaenda*. However, *S. dehiscens* can be diagnosed by having radial and inner tangential walls of exotesta cells with finely verrucose appearance and small pits (Puff et al., 1993). In all analyses presented here, this species is not nested within any of the well-circumscribed Mussaendeae genera we recognize here. Accordingly, we continue to maintain its current generic status.

Neomussaenda—We are unable to get good material for *Neomussaenda*; our efforts to get DNA from herbarium specimens were repeatedly unsuccessful. *Neomussaenda* was originally described by Tange (1994) to accommodate the species of the genus *Greenea*, *G. xanthophytoides*, and his new species, *N. kostermansiana*. *Neomussaenda* can be distinguished from the remaining Mussaendeae genera by a combination of induplicate-valvate aestivation, seed exotestal cells with tuberculate inner wall, idioblasts filled with numerous minute druses, and drupaceous fruits. Tange (1994) argued that *Neomussaenda* is closely related to *Pseudomussaenda* and *Schizomussaenda* based on the following characters: bifid stipules, terminal thyrsoidal inflorescences, induplicate-valvate aestivation, and fruits with a splitting zone opposite the placenta. Such relationship is inconsistent with the morphological tree shown in Andersson (1996, p. 154), which placed *Neomussaenda* with high support, as sister to the rest of Mussaendeae sensu Bremer and Thulin (1998). Tange's hypothesis is also not supported by our results from the combined data sets as *Pseudomussaenda* is resolved as closely related to the Afro-Asian *Mussaenda* clade. We continue to maintain the current status of *Neomussaenda* until new data are available. All accepted genera and their synonymies, distribution, and number of species are given in Table 4.

Evolution and phylogenetic utility of some morphological features for Mussaendeae and Mussaenda s.s.—*Life forms*—

There is a great variation of habit in Mussaendeae sensu Bremer and Thulin (1998) ranging from erect shrubs or trees to scandent or scrambling shrubs, true lianas or small suffrutices. All *Bremeria*, *Landiopsis*, *Neomussaenda*, and *Schizomussaenda* are typically erect shrubs or trees (e.g., some of the Malagasy *Bremeria* species). In contrast, both scandent or scrambling, and erect shrubs are commonly found in our newly delimited *Mussaenda*, with only few true lianas and two suffrutescent species (*M. uniflora* and *M. parva*). Both scandent and erect shrub habits are found in *Heinsia*. Therefore, the taxonomic usefulness of life forms in Mussaendeae is rather limited.

Breeding systems—The breeding system of Mussaendeae sensu Bremer and Thulin is predominantly heterodistylous. However, some African (e.g., *M. chippi* and *M. tristigmatica*, Hallé, 1961) and Asian (e.g., *M. parva*, *M. parvifolia*, *M. reinwardtiana*, *M. uniflora*, Bremekamp, 1937; Puff et al., 1993) *Mussaenda* are functionally dioecious, suggesting that dioecy in the newly circumscribed *Mussaenda* has evolved from distyly. Puff et al. (1993) argue that heterodistyly in some Afro-Asian *Mussaenda* species (e.g., *M. sanderiana*) and *Schizomussaenda dehiscens* may not always be stable. Their observations show that the anthers and stigmas of these plants are not clearly separated spatially, indicating a possible reversal to homostyly. Based on the evidence described, we conclude that breeding systems of *Mussaenda* are evolutionarily labile. In contrast, *Bremeria*, *Heinsia*, *Neomussaenda*, and *Pseudomussaenda* are invariably heterodistylous.

Corolla aestivation types—Both imbricate (*Heinsia* and *Landiopsis*) and valvate aestivations are found within Mussaendeae sensu Bremer and Thulin. There are three types of valvate aestivations: induplicate- (*Neomussaenda*, *Pseudomussaenda*, and *Schizomussaenda*), reduplicate- (the newly delimited *Mussaenda*), and induplicate- and reduplicate-valvate (*Bremeria*) aestivations. Our findings show that both imbricate and induplicate-valvate aestivations have evolved independently at least two times within Mussaendeae sensu Bremer and Thulin, indicating that they should not be used alone for diagnosing genera in this tribe. In contrast, the reduplicate- and induplicate-reduplicate-valvate aestivations evolved only once within the tribe, making them reliable characters for diagnosing our newly circumscribed *Mussaenda* and *Bremeria*, respectively.

Semaphylls—About 67% of the members of Mussaendeae have developed enlarged, petaloid calyx lobes, which probably function as optical organs for attracting nectar or pollen-feeding insects from long distances. The presence and/or absence of semaphylls were traditionally used as primary criterion for delimiting *Mussaenda* (e.g., Bremekamp, 1937). Our studies clearly show that semaphylls have evolved independently numerous times within Mussaendeae sensu Bremer and Thulin (1998) and the newly delimited *Mussaenda*. On the other hand, it is worth noting that 98% of our newly circumscribed *Mussaenda* have enlarged calyx lobes. Also, all Mussaendeae genera with dry, capsular fruits (*Landiopsis*, *Pseudomussaenda*, and *Schizomussaenda*) have semaphylls.

Fruit types—Fruit types were used to segregate *Aphaenandra*, *Pseudomussaenda*, and *Schizomussaenda*, all with dry capsular fruits, from *Mussaenda* s.l. with fleshy, indehiscent fruits. Our results, however, indicate that fleshy fruits have evolved independently at least five times and capsular fruits at least three times within Mussaendeae sensu Bremer and Thulin. Therefore, fruit types should not be used as primary character for recognizing genera in this tribe; on the other hand, they can be used to characterize genera of Mussaendeae in combination with other characters. The drupaceous fruit is only found in *Neomussaenda* and is a good character for recognizing this genus.

Biogeography of Mussaenda s.s.—The biogeographical history of the newly circumscribed *Mussaenda* s.s. can be inferred based on the results presented in this study. Our combined tree (Fig. 3) strongly suggests an African origin of *Mussaenda* s.s., which appears to have started to diversify in mainland Africa, where a total of 35 species is currently present. The Asian *Mussaenda* species seem to have descended from an African progenitor that must have reached Asia via a long-distance dispersal event. The major radiations of *Mussaenda* s.s. seems to have occurred only after the group began to colonize Asia, where ca. 97 species (73.48%) of the 132 *Mussaenda* are presently found. Despite the fact that the Afro-Asian *Mussaenda* species are shown to be closely related (Figs. 1–3), mainland Africa and Asia do not share in common any *Mussaenda* species. On the other hand, the most widespread African *Mussaenda* species, *M. arcuata*, is the only African *Mussaenda* species that has successfully reached the Comoro islands, Madagascar, and the Mascarenes, probably via stepping-stone dispersal.

Synopsis—*Mussaenda* Burm. ex L. in Sp. Pl.: 177 (1753); Gen. Pl. ed. 5: 85 (1754). TYPE: *Mussaenda frondosa* L. (lectotype, designated by Jayaweera (1963: 239), *Hermann s.n.*, BM).
  Aphaenandra Miq., in Fl. Ned. Ind. 2: 341 (1857); in Blumea, Suppl. 1, 120 (1937). TYPE: *Aphaenandra sumatrana* Miq.
  Landia Comm. ex Juss., in Gen. Pl. 201 (1789). TYPE: not designated.
  Shrubs to small trees, scandent shrubs, lianas, or rarely suffrutices. Leaves opposite, decussate, small to large, petiolate or rarely subsessile; blades ovate or elliptic, usually pubescent especially on the midrib and veins underneath; stipules bifid, persistent or deciduous, with few or many colleters in continuous rows and/or in groups of two at the base. Inflorescences terminal cymose corymbs, glabrous or variously hairy, few to many-flowered or rarely reduced to a single flower; bracts and bracteoles few to numerous, entire or trilobed (lateral lobes always shorter); flowers small, typically heterostylous, usually 5-merous, (sub)sessile or shortly pedicellate; calyx tubes extremely reduced or cup-shaped to shortly tubular or ovoid, usually pubescent, the lobes extremely short to long, linear to lanceolate or ovate, rarely foliaceous, occasionally with a single semaphyll, rarely absent or all developed into semaphylls (e.g., *M. philippica* var. *aurorae*); colleters frequently in sinuses between calyx lobes; semaphylls white to creamy yellow or, rarely, red, elliptic to ovate or orbicular; corolla tubes short, cylindrical or infundibular, usually forming a distinctly swollen part around anthers, glabrous or pubescent outside and with unicellular trichomes inside, the lobes reduplicate-valvate in bud, spreading at anthesis, orange, yellow to red, or rarely white, elliptic to ovate, rarely linear-lanceolate, abaxially pubescent and adaxially papillate, apical filiform appendages

usually present; stamens inserted immediately below the opening or above the middle of the tube in short-styled morphs and around the middle in long-styled morphs; filaments short, anthers 5, included, bilobed at base, dorsifixed near base; 2-carpellate ovaries, rarely 3–4 carpels; placentae peltate; ovules numerous, imbedded in fleshy placentae; styles slender, typically glabrous; stigma lobes bifid, included or semi-exserted in long-styled morphs. Fruits fleshy, indehiscent berry-like, ellipsoid, obovoid to globose, glabrous or pubescent, calyx lobes deciduous or persistent, warts present or rarely absent; seeds numerous, endospermic; exotesta cells polygonal, outer tangential walls thin and ± smooth, radial and inner tangential walls thickened with large pits.

Number of species: 132 species (97 species in tropical Asia and 35 species in mainland Africa).

Diagnostic characters: *Mussaenda* s.s. can easily be diagnosed by having smaller flowers (than *Bremeria*), 3–5(–8) centimeter long, with reduplicate-valvate aestivation.

Bremeria Razafim. and Alejandro, gen. nov. TYPE: *Bremeria landia* (Poir.) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda landia* Poir., in Lam. Encycl. 4: 392 (1797); D. C., Prodr. 4: 372 (1830); Bojer, H.M.: 165 (1837); Bak., F.M.S.: 140 (1877); Cordem., F.R.: 503 (1895); R.E. Vaugham, Mauritius Inst. Bull. 1: 46 (1937). TYPE: Mauritius, *Commerson s.n.* (syntype, P–LA; isosyntype, P).

Bremeria ab aliis generibus Mussaendeae facile distincta est aestvatione valvata-induplicata combinata cum aestivatione valvata-reduplicata, stylis dense puberulis.

Shrubs to medium-sized trees. Leaves opposite, decussate, usually pubescent and sometimes scabrous; stipules bifid, sometimes divided to the base, pubescent on both sides or only outside, deciduous, the colleters few or many and usually in groups of two at the base or extending in or above the middle. Inflorescences typically terminal, paniculate, sometimes reduced to a single flower; bracts and bracteoles present or, rarely, absent, usually entire or bilobed; flowers usually large, 5-merous, short to long pedicellate; calyx tubes oblong or ovoid, variously hairy, rarely glabrous, the lobes long-linear to subulate, mostly unequal, pubescent on both sides or rarely glabrous inside, often persistent; colleters few to numerous, usually between sinuses or sometimes along the margins of calyx lobes; corolla tubes long, funnel-shaped, always evenly pubescent all over outside and with unicellar trichomes inside, lobes with both induplicate-valvate and reduplicate-valvate aestivation in bud, spreading at anthesis, white to pink, or greenish at the base and at the top and reddish in the middle (*Bremeria landia*), usually abaxially pubescent and adaxially tomentose, apical filiform appendages typically present; stamens inserted at the throat or to midway on the tubes, filaments short, anthers included; ovaries 2-carpellate, styles slender, typically pubescent, stigmas subentire or shortly bilobed, always included; ovules many per locule. Fruits large, fleshy, indehiscent berries or drupes, always crowned by the persistent calyx lobes; seeds numerous, with endosperm, thickened, pitted.

Number of species: 24 species in Madagascar and four species in the Mascarenes.

Diagnostic characters: *Bremeria* differs from *Mussaenda* s.s. by its large flowers, 7–13(–15) cm long, without petaloid calyx lobes, induplicate-reduplicate-valvate aestivation and densely pubescent styles.

New combinations—Here, we present our 19 new combinations that consist of 18 described Malagasy and one Mascarene *Mussaenda* species. All necessary lectotypifications of *Bremeria* species will be published in the ongoing systematic revision of the genus (Andriambololonera and Razafimandimbison, unpublished manuscript).

Bremeria asperula (Wernham) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda aperula* Wernham, in J. Bot. 52: 67 (1914). TYPE: Madagascar, *Baron 493* (syntype, BM, P).

Bremeria decaryi (Homolle) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda decaryi* Homolle, in Not. Syst. (Paris) 7: 3 (1938). TYPE: Madagascar, Domaine oriental, Mont de Vatovavy, *Perrier de la Bâthie 3988, Perrier de la Bâthie 3994*; *Decary 4908, Decary 5455, Decary 4872, Decary 5562* (syntypes, P).

Bremeria erectiloba (Wernham) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda erectiloba* Wernham, in J. Bot. 52: 67–68 (1914). TYPE: Madagascar, Tanala, Ambohimitombo forest, *Deans Cowan s.n.*; *Forsyth Major 274* (syntypes, BM, K, P; isosyntype, MO).

Bremeria fusco-pilosa (Baker) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda fusco-pilosa* Baker, in J. Linn. Soc., Bot. 21: 410 (1885). TYPE: Madagascar, *Baron 2467, Baron 2470, Baron 6118* (syntypes, K, P).

Bremeria gerrardi (Homolle) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda gerrardi* Homolle, in Not. Syst. (Paris) 7: 4 (1938). TYPE: Madagascar, *Gerrard 21–6166, no. 37* (syntype, K).

Bremeria humblotii (Wernham) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda humblotii* Wernham, in J. Bot. 52: 70 (1914). TYPE: Madagascar, *Humblot 617* (syntype, K, P).

Bremeria hymenopogonoides (Baker) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda hymenopogonoides* Baker, in J. Bot. 20: 138 (1882). TYPE: Madagascar, forests of the Tanala country, *Baron 313* (holotype, K; isotype, P).

Bremeria lantziana (Homolle) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda lantziana* Homolle, in Not. Syst. (Paris) 7: 4 (1938). TYPE: Madagascar, Domaine oriental, Matatane, *Lantz s.n.*; *Decary 10999* (syntypes, P).

Bremeria latisepala (Homolle) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda latisepala* Homolle, in Not. Syst. (Paris) 7: 5 (1938). TYPE: Madagascar, *Expos. Colon. Marseille s.n.* (syntype, P).

Bremeria mauritiensis (Wernham) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda mauritiensis* Wernham, in J. Bot. 52: 66–67 (1914). TYPE: Mauritius, in sylvis, ad radices montium; Sur les hautes montagnes, *Bojer s.n.*; *Blackburn s.n.* (syntypes, K).

Bremeria monantha (Wernham) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda monantha* Wernham, in J. Bot. 52: 70 (1914). TYPE: Madagascar, between Tamatave and Antananarivo, *Meller s.n.*; *Thompson s.n.* (syntypes, BM, K).

Bremeria perrieri (Homolle) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda perrieri* Homolle, in Not. Syst. (Paris) 7: 5 (1938). TYPE: Madagascar, Domaine oriental, rivière Anove, côte Est, *Perrier de la Bâthie 3753*; *Decary 131* (syntypes, P).

Bremeria pervillei (Wernham) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda pervillei* Wernham, in J. Bot. 52: 67 (1914). TYPE: Madagascar, *Baron 6373, Baron 5800*; *Hildebrandt 3003* (syntypes, P).

Bremeria pilosa (Baker) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda pilosa* Baker, in Kew. Bull. 105 (1895). TYPE: Madagascar, *Baron 6179* (syntypes, K, P).

Bremeria punctata (Drake) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda punctata* Drake, in Grandidier, Hist. Pl. Madagascar t. 36: 447 (1897). TYPE: Madagascar, Mahalougouloue?, *Thompson s.n.* (syntype, BM).

Bremeria ramosissima (Wernham) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda ramosissima* Wernham, in J. Bot. 52: 69 (1914). TYPE: Madagascar, *Humblot 392* (syntypes, K, P).

Bremeria scabridior (Wernham) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda scabridior* Wernham, in J. Bot. 52: 71 (1914). TYPE: Madagascar, *Baron 1505, Baron 3975* (syntypes, K).

Bremeria trichophlebia (Baker) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda trichophlebia* Baker, in J. Linn. Soc., Bot. 20: 166 (1882–1883). TYPE: Madagascar, *Baron 493* (syntypes, K, P).

Bremeria vestita (Baker) Razafim. and Alejandro, comb. nova. Basionym: *Mussaenda vestita* Baker, in J. Linn. Soc., Bot. 20: 166 (1882–1883). TYPE: Madagascar, Betsileo-land, *Baron 55*; *Langley-Kitching s.n.* (syntypes, K).

In conclusion, the present phylogenetic studies highly support the polyphyly of *Mussaenda* s.l. as currently circumscribed, whereas the monophyly of Mussaendeae sensu Bremer and Thulin (1998) is further supported. We describe a new genus *Bremeria* to accommodate the Malagasy and Mascarene *Mussaenda* species, merge *Aphaenandra* in *Mussaenda* s.s., which is now restricted to include only the African and Asian *Mussaenda* species. The newly circumscribed Mussaendeae contains seven genera: *Bremeria*, *Heinsia*, *Landiopsis*, *Mussaenda* s.s., *Neomussaenda*, *Pseudomussaenda*, and *Schizomussaenda* (Table 4). Many of the vegetative and reproductive characters traditionally used to delimit genera in Mussaendeae sensu Bremer and Thulin (1998) are shown to be unreliable for group recognition because they have evolved independently several times within the tribe or even within *Mussaenda* s.s. However, some of them still can be used in combination with other characters to characterize genera. Our results suggest an African origin of both the newly delimited *Mussaenda* s.s. and the Asian *Mussaenda*.

LITERATURE CITED

ANDERSSON, L. 1996. Circumscription of the tribe Isertieae (Rubiaceae). *Opera Botanica Belgica* 7: 139–164.

ANDERSSON, L., AND J. H. E. ROVA. 1999. The *rps16* intron and the phylogeny of the Rubioideae (Rubiaceae). *Plant Systematics and Evolution* 214: 161–186.

ANDREASEN, K., B. BALDWIN, AND B. BREMER. 1999. Phylogenetic utility of the nuclear rDNA ITS region in subfamily Ixoroideae (Rubiaceae): comparisons with cpDNA *rbcL* sequence data. *Plant Systematics and Evolution* 217: 119–135.

BAILLON, H. 1880. Histoire des plantes, vol. 7. Hachette, Paris, France.

BALDWIN, B. G., M. J. SANDERSON, J. M. PORTER, M. F. WOJCIECHOWSKI, C. S. CAMPBELL, AND M. J. DONOGHUE. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* 82: 247–277.

BOSSER, J., AND D. LOBREAU-CALLEN. 1998. *Landiopsis* Capuron ex Bosser, genre nouveau de Rubiaceae de Madagascar. *Adansonia* 20: 131–137.

BREMEKAMP, C. E. B. 1937. Notes on the Rubiaceae of tropical Asia. *Blumea* 1Supplement: 112–122.

BREMEKAMP, C. E. B. 1947. A monograph of the genus *Acranthera* Arn. ex Meisn. *Journal of the Arnold Arboretum* 28: 261–308.

BREMEKAMP, C. E. B. 1966. Remarks on the position, the delimitation and the subdivision of the Rubiaceae. *Acta Botanica Neerlandica* 15: 1–33.

BREMER, B. 1996. Combined and separate analyses of morphological and molecular data in the plant family Rubiaceae. *Cladistics* 12: 21–40.

BREMER, B., K. ANDREASEN, AND D. OLSSON. 1995. Subfamilial and tribal relationships in the Rubiaceae based on *rbcL* sequence data. *Annals of the Missouri Botanical Garden* 82: 383–397.

BREMER, B., R. J. JANSEN, B. OXELMAN, M. BACKLUND, H. LANTZ, AND K.-J. KIM. 1999. More characters or more taxa for a robust phylogeny—case study from the coffee family (Rubiaceae). *Systematic Biology* 48: 413–435.

BREMER, B., AND J. F. MANEN. 2000. Phylogeny and classification of the subfamily Rubioideae (Rubiaceae). *Plant Systematics and Evolution* 225: 43–72.

BREMER, B., AND M. THULIN. 1998. Collapse of Isertieae, re-establishment of Mussaendeae, and a new genus of Sabiceeae (Rubiaceae); phylogenetic relationships based on *rbcL* data. *Plant Systematics and Evolution* 211: 71–92.

BRIDSON, D. M., AND B. VERDCOURT. 1988. Isertieae. *In* R. M. Polhill [ed.], Flora of tropical East Africa, Rubiaceae, part 2. 460–475. A. A. Balkema, Rotterdam, Netherlands.

CANDOLLE, A. P. DE. 1830. Prodromus systematis naturalis regni vegetabilis 4. Treutell & Würtz, Paris, France.

CHASE, M. W., AND H. H. HILLS. 1991. Silica gel: an ideal material for preservation of leaf samples for DNA studies. *Taxon* 40: 215–220.

CRAIB, W. G. 1932. Florae Siamensis Enumeratio. A list of the plants known from Siam with notes of their occurrence. Caprifoliaceae & Rubiaceae (in part), vol. 2, 1–145. Siam Society, Bangkok, Thailand.

DE VOOGD, C. N. A. 1929. *Aphaenandra. De Tropische Natuur* 18: 110.

DON, G. 1834. A general history of the dichlamydeous plants, vol. 3, Calyciflorae: viii + 867. London, UK.

FARRIS, J. S. 1989. The retention index and the rescaled consistency index. *Cladistics* 5: 417–419.

FARRIS, J. S., M. KÄLLERSJÖ, A. G. KLUGE, AND C. BULT. 1995. Testing significance of incongruence. *Cladistics* 10: 315–319.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evolution* 39: 783–791.

GOLDMAN, N., J. P. ANDERSON, AND A. G. RODRIGO. 2000. Likelihood-based tests of topologies in phylogenetics. *Systematic Biology* 49: 652–670.

HALLÉ, N. 1961. Contribution à l'étude biologique et taxonomique des Mussaendeae (Rubiaceae) d'Afrique tropicale. *Adansonia* 1: 266–298.

HOOKER, J. D. 1873. Ordo LXXXIV, Rubiaceae. *In* G. Bentham and J. D. Hooker [eds.], Genera plantarum ad exemplaria imprimis in herbariis kewensibus servata defirmata, vol. 2, 7–151. Lovell Reeve, London, UK.

HOOKER, J. D. 1880. Flora of British India, vol. 3, Mussaendeae, 86–92. Lovell Reeve, London, UK.

JOCHEMS, S. C. J. 1929. *Aphaenandra. De Tropische Natuur* 18: 153.

KLUGE, A. G., AND J. S. FARRIS. 1969. Quantitative phyletics and the evolution of anurans. *Systematic Zoology* 18: 1–32.

KURZ, S. 1887. Forest Flora of British Burma, vol. 2, *Mussaenda*, 55–58. M/S Bishen Singh Mahendra Pal Singh, Dehra Dun, India and M/S Periodical Experts, Delhi, India.

LEPPIK, E. E. 1977. Calyx-borne semaphylls in tropical Rubiaceae. *Phytomorphology* 27: 161–168.

LI, H. L. 1943. *Schizomussaenda*, a new genus of the Rubiaceae. *Journal of the Arnold Arboretum* 24: 99–102.

MABBERLEY, D. J. 1997. The plant-book—a portable dictionary of the vascular plants, 2nd ed. Cambridge University Press, Cambridge, UK.

MADDISON, D. R. 1991. The discovery and importance of multiple islands of most-parsimonious tress. *Systematic Zoology* 40: 315–328.

MEISNER, C. D. F. 1838. Plantarum vascularium genera secundum ordines naturales digesta 1, *Acranthera*, 162. Leipzig, Germany.

MEVE, U., AND S. LIEDE. 2002. A molecular phylogeny and generic rearrangement of the stapelioid Ceropegieae. *Plant Systematics and Evolution* 234: 171–209.

MEVE, U., AND S. LIEDE. 2004. Subtribal division of Ceropegieae. *Taxon* 53: 61–72.

MIQUEL, F. A. W. 1857. Flora van Nederlandsch-Indië, vol. 2, *Aphaenandra*, 341–342. Amsterdam, Netherlands.

NAIKI, A., AND M. KATO. 1999. Pollination system and evolution of dioecy from distyly in *Mussaenda parviflora* (Rubiaceae). *Plant Species Biology* 14: 217–227.

NYLANDER, J. A. 2002. Mrmodeltest, version 1.1b. [Software program dis-

tributed online by the author]. Department of Systematic Zoology, EBC, Uppsala University, Sweden, website, http://www.ebc.uu.se/systzoo/staff/nylander.html.

PERSSON, C. 2000. Phylogeny of the Neotropical *Alibertia* group (Rubiaceae), with emphasis on the genus *Alibertia*, inferred from ITS and 5S ribosomal DNA sequences. *American Journal of Botany* 87: 1018–1028.

POPP, M., AND B. OXELMAN. 2001. Inferring the history of the polyploid *Siline aegaea* (Caryophyllaceae) using plastid and homoeologous nuclear DNA sequences. *Molecular Phylogenetics and Evolution* 20: 474–481.

PUANGSOMLEE, P., AND C. PUFF. 2001. Chromosome numbers of Thai Rubiaceae. *Nordic Journal of Botany* 21: 165–175.

PUFF, C., AND A. IGERSHEIM. 1994. The character states of *Mussaendopsis* Baill. (Rubiaceae-Coptosapelteae). *Flora* 189: 161–178.

PUFF, C., A. IGERSHEIM, AND U. ROHRHOFER. 1993. *Pseudomussaenda* and *Schizomussaenda* (Rubiaceae): close allies of *Mussaenda*. *Bulletin de Jardin Botanique National de Belgique* 62: 35–68.

RAZAFIMANDIMBISON, S. G., AND B. BREMER. 2001. Tribal delimitation of Naucleeae (Cinchonoideae, Rubiaceae): inference from molecular and morphological data. *Systematics and Geography of Plants* 71: 515–538.

RAZAFIMANDIMBISON, S. G., AND B. BREMER. 2002. Phylogeny and classification of Naucleeae s.l. (Rubiaceae) inferred from molecular (ITS, *rbcL* and *trnT-F*) and morphological data. *American Journal of Botany* 89: 1027–1041.

ROBBRECHT, E. 1988. Tropical woody Rubiaceae. *Opera Botanica Belgica* 1: 1–271.

ROVA, J. H. E., P. DELPRETE, L. ANDERSSON, AND V. ALBERT. 2002. A *trnL-F* cpDNA sequence study of the Condamineeae-Rondeletieae-Sipaneeae complex with implications on the phylogeny of the Rubiaceae. *American Journal of Botany* 89: 145–159.

SCHUMANN, K. 1891. Rubiaceae. *In* A. Engler and K. Prantl [eds.], Die natürlichen Pflanzenfamilien, vol. 4, 1–156. Engelmann, Leipzig, Germany.

SCHUMANN, K. 1897. Rubiaceae. *In* A. Engler and K. Prantl [eds.], Die natürlichen Pflanzenfamilien, Nachträge [I] zum II–IV, 309–316. Engelmann, Leipzig, Germany.

SHIMODAIRA, H., AND M. HASEGAWA. 1999. Multiple comparisons of log-likelihoods with application to phylogenetic inference. *Molecular Biology and Evolution* 16: 1114–1116.

SIMMONS, M. P., AND H. OCHOTERENA. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Sytematic Biology* 49: 369–381.

SWOFFORD, D. L. 2000. PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4.0b. Sinauer Associates, Sunderland, Massachusetts, USA.

TANGE, C. 1994. *Neomussaenda* (Rubiaceae), a new genus from Borneo. *Nordic Journal of Botany* 14: 495–500.

WERNHAM, H. F. 1914. The Mussaendas of Madagascar. *The Journal of Botany* 52: 64–72.

WERNHAM, H. F. 1916. *Pseudomussaenda*: a new genus of Rubiaceae. *The Journal of Botany* 54: 297–301.