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Re-assessment of monophyly, evolution of myrmecophytism, and rapid radiation in *Neonauclea* s.s. (Rubiaceae)

Sylvain G. Razafimandimbison[a,b,*], Joachim Moog[c], Henrik Lantz[a], Ulrich Maschwitz[c], Birgitta Bremer[a,b]

[a] *Department of Systematic Botany, Evolutionary Biology Centre, Uppsala University, Norbyvägen 18 D, SE-752 36, Uppsala, Sweden*
[b] *The Bergius Foundation at the Royal Swedish Academy of Sciences, P.O. Box 50017, SE-104 05, Stockholm, Sweden*
[c] *Department of Zoology, University of Frankfurt, Siesmayerstr. 70, D-60054 Frankfurt, Germany*

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Abstract

The biologically interesting ant–plant association, myrmecophytism, occurs in ca. 140 of the 11,000 species and 22 of the 630 genera of the coffee family (Rubiaceae). These myrmecophytic Rubiaceae species are predominantly distributed in Southeast Asia, especially the Malesian region, with comparatively few species in mainland Africa and the Neotropics. The mostly Southeast Asian genus *Neonauclea* s.s is one of the three Rubiaceae genera with extensive radiation of myrmecophytes and also the most speciose genus of the tribe Naucleeae s.l. We perform parsimony phylogenetic analyses of *Neonauclea* s.s., previously resolved as paraphyletic, and its allied genera using both ETS and ITS sequencing data to test: (1) the paraphyly of *Neonauclea* s.s.; (2) the phylogenetic relationships within the *Ludekia–Myrmeconauclea–Neonauclea* complex; and (3) the evolution of myrmecophytism within the complex. The earlier proposed paraphyly of *Neonauclea* s.s. appears to be the result of the combined effects of parallel substitutions in *Metadina trichotoma* and the sampled ITS putative pseudogenes of *Neonauclea longipedunculata* and losses of some synapomorphies of *Neonauclea* s.s. in the latter. The analyses present strong support for the monophyly of *Myrmeconauclea* and *Neonauclea* s.s. and their sister-group relationships. Our findings additionally favor the hypothesis of multiple origins of myrmecophytism in the Bornean *Neonauclea*, which have independently been exploited by at least three *Cladomyrma* ant species. Furthermore, we interpret the low levels of variation in both the ETS and ITS sequences as indication of a recent and rapid radiation for *Neonauclea* s.s. (with 65 species) and a recent and slow radiation for *Myrmeconauclea* (with three species). We argue that the rapid diversification of *Neonauclea* s.s. is partly associated with the nature of its fruits and its ability to colonize a wide range of habitats. We postulate that both ecological and geographical events may have been responsible for the radiation of the non-myrmecophytic *Neonauclea* species. Finally, we argue that the acquisition of the pseudo-multiple fruits and long-tailed seeds has allowed *Myrmeconauclea* to specialize on rheophytic habitats but its narrow ecological tolerance may have hindered its speciation.

Keywords: ETS; ITS; *Myrmeconauclea*; Myrmecophytism; Naucleeae s.l.; *Neonauclea* s.s.; Putative pseudogenes; Rapid diversification; Recent hybridization

1. Introduction

Both the circumscriptions and generic limits of the tribe Naucleeae of the subfamily Cinchonoideae (Rubiaceae or coffee family) have changed as a result of the recent phylogenetic studies based on the combined morphological-molecular (ITS, *rbc*L, and *trn*T-F) datasets (Razafimandimbison and Bremer, 2001, 2002). Naucleeae as currently circumscribed comprises 179 species and 24 genera that are placed in six major groups, formally recognized as subtribes (Razafimandimbison and Bremer, 2002). The genus *Neonauclea* sensu stricto (s.s.)

* Corresponding author. Fax: +46 (0) 8 16 55 25.
E-mail address: sylvain.razafimandimbison@bergianska.se (S.G. Razafimandimbison).

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(Ridsdale, 1989) of the subtribe Adininae sensu Razafimandimbison and Bremer (2002) is the most speciose with 65 species of trees and shrubs. The genus was indicated to be paraphyletic in Razafimandimbison and Bremer (2001, 2002). The ITS clonal sequence of *Neonauclea longipedunculata* (hereinafter *N. longipedunculata*) and *N. clemensii* did not form a monophyletic group in the ITS tree of Razafimandimbison and Bremer (2001). The former was resolved with weak support as sister to the Southeast Asian monotypic genus *Metadina trichotoma*; the latter and *Myrmeconauclea strigosa* formed a strongly supported monophyletic group. In Razafimandimbison and Bremer (2002), *Neonauclea* s.s., represented by four species, *N. brassii*, *N. clemensii*, *N. forsteri*, and *N. longipedunculata*, formed a strongly (JK = 94 and BS = 89, Fig. 3: 1037) supported monophyletic group with *Ludekia* and *Myrmeconauclea*. The position of *Ludekia*, represented by the type species *L. borneensis*, was unresolved and *Myrmeconauclea*, represented by the type species *M. strigosa*, *N. clemensii*, and *N. longipedunculata* formed a moderately supported subclade. Sequencing a second variable and informative marker was necessary to test the earlier proposed paraphyly of *Neonauclea* s.s. The level of variation found between the ITS sequences of *Ludekia*, *Myrmeconauclea*, and *Neonauclea* s.s. in Razafimandimbison and Bremer (2001, 2002) indicates that combining the ITS dataset with another dataset from a fast-evolving marker (e.g., external transcribed spacers, ETS, of the rDNA region) might provide enough resolution for assessing phylogenetic relationships within *Neonauclea* s.s. and between the three genera. Sequence data from the ETS region have been shown to be useful for inferring phylogenetic relationships of closely related species in various Angiosperm groups (e.g., Baldwin and Markos, 1998), including Rubiaceae (e.g., Negrón-Ortiz and Watson, 2002; Markey and de Lange, 2003; Nepokroeff et al., 2003). On the other hand, sequences of *Ludekia*, *Myrmeconauclea*, and *Neonauclea* from the *rbc*L (Razafimandimbison and Bremer, 2001, 2002) and *trn*T-F (Razafimandimbison and Bremer, 2002) regions are almost without variation and are not suitable markers for inferring phylogenetic relationships in the *Ludekia–Myrmeconauclea–Neonauclea* complex. The present study focuses on this predominantly Southeast Asian group, which contains 70 (ca. 39%) of the 179 Naucleeae species (Ridsdale, 1978).

The two *Ludekia* species (*L. bernardoi* and *L. borneensis*) are restricted to the Philippines (Leyte, Luzon, Masbates, Mindanao, and Samar Islands) and Borneo, respectively, and are primarily distributed in evergreen rainforests. On the other hand, the two *Myrmeconauclea* species (*M. strigosa* and *M. stipulacea*) and *M. rheophila*, respectively, are confined to Borneo and Anambas Island (Indonesia). These species are rheophytic shrubs that grow on the banks of creeks or rivers and/or in places subject to overflow in times of heavy rain (Merrill, 1920). Finally, *Neonauclea* s.s. has a wider geographic range extending from India to Vietnam through the Malesian archipelago to northern Australia (Fig. 1). The center of diversity of the genus is Borneo, New Guinea, the Philippines, and Sulawesi. *Neonauclea* species grow on various types of habitats ranging from evergreen rainforests, deciduous, dry forests to swamped forests. According to Ridsdale (1989), four *Neonauclea* species (*N. angustifolia*, *N. charmersii*, *N. jagori*, and *N. pallida*) are rheophytes. Many of the *Neonauclea* species (e.g., *N. gigantea*, *N. longipedunculata*, *N. paracyrtopoda*) included in this study are pioneer trees that often colonize open areas such as new clearings, landslides, new roadcuts, and secondary forests (Moog and Maschwitz, unpubl. data). The diagnostic morphological characters of the three genera are summarized in Table 1. In both *Myrmeconauclea* and *Neonauclea* s.s., the calyx lobes are always prolonged by well-developed appendages that conceal the young corollas (see Ridsdale, 1989: Fig. 3; Plates 1–15). These appendages break off as the flower buds continue their growth. In contrast, they are absent in *Ludekia* and the other Naucleeae genera. Both *Ludekia* and *Neonauclea* s.s. and the other Adininae genera produce capsular fruits that dehisce septicidally and loculicidally into four parts from base to apex. Their seeds are bilaterally compressed and shortly winged at both ends (Ridsdale, 1978). In contrast, all three *Myrmeconauclea* species produce what appear to be false multiple fruits, called pseudosyncarps by Ridsdale (1978) and pseudo-multiple fruits by Razafimandimbison and Bremer (2002), because only the upper parts of adjacent individual fruits are connate. The fused tissues break apart when the infructescences are fully mature (Ridsdale, 1978). *Myrmeconauclea* fruits bear many small, fusiform seeds with long-ventral tails attached at one end (Merrill, 1920; Ridsdale, 1978). Merrill (1920, 375–376) observed that "the persistent tips of the calyx segments fall, leaving a small perforation at the apex of each individual fruit." His assumption that the mature seeds escape through the perforation is consistent with their shape and size. Based on this hypothesis, the fruits of *Myrmeconauclea* could be considered to be capsules.

Myrmecophytism, ant–plant association, has been recorded from ca. 140 (1.3%) of the 11,000 species and 22 (3.5%) of the 630 genera of the coffee family (Rubiaceae) [total numbers of Rubiaceae species and genera according to Robbrecht (1996)]. However, only three of the 22 Rubiaceae genera (*Hydnophytum*, *Myrmecodia*, and *Neonauclea* s.s.) are known to have extensive radiations of myrmecophytic species (see Table 2). These Rubiaceae myrmecophytic species are presently classified in 11 different tribes and in all three subfamilies (see Table 2), indicating multiple origins of myrmecophytism within the family. They are predominantly distributed in Southeast Asia, with only a few species in mainland Africa and

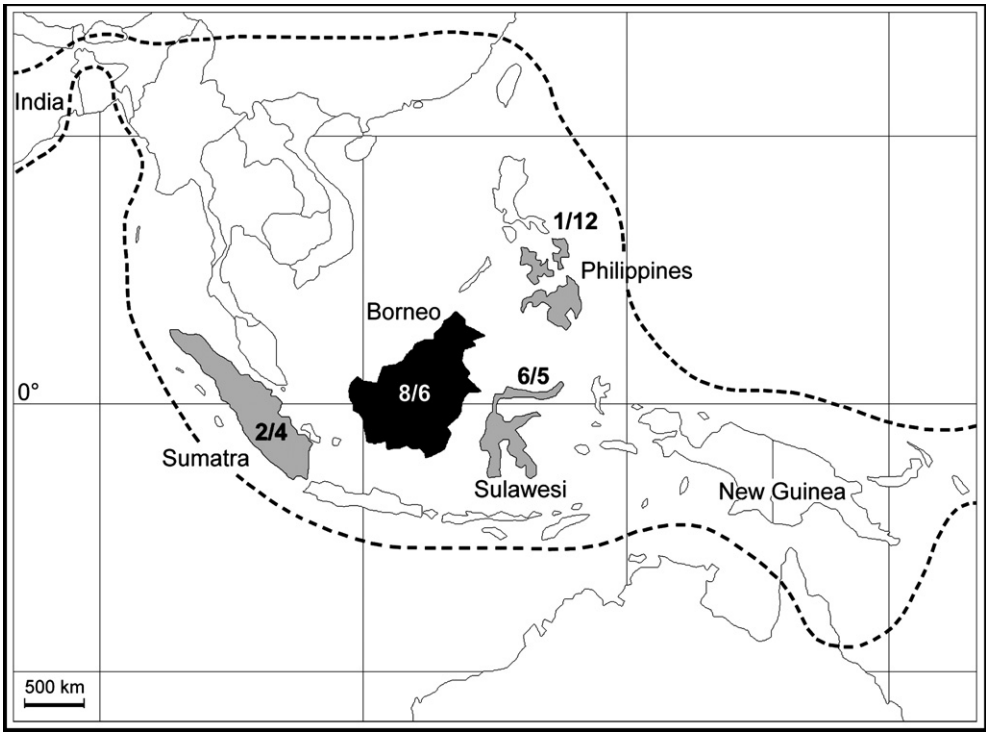

Fig. 1. Geographic distribution of *Neonauclea* s.s. Black and gray areas denote the range of myrmecophyte *Neonauclea* inhabited by *Cladomyrma* and *Crematogaster* ants, respectively; ratios represent the number of myrmecophytic/nonmyrmecophytic *Neonauclea* species for that given area; dashed line delimits the current distribution of *Neonauclea* s.s.

Table 1
Diagnostic morphological characters of *Ludekia*, *Myrmeconauclea*, and *Neonauclea* s.s.

Characters	*Ludekia*	*Myrmeconauclea*	*Neonauclea* s.s.
Calyx lobes	Never prolonged by well-developed appendages	Always prolonged by well-developed appendages that always conceal young corollas	Always prolonged by well-developed appendages that always conceal young corollas
Stigmas	Globose with prominent longitudinal ridges	Globose, smooth	Globose, smooth
Infructescences	Adjacent fruits completely free from each other	Adjacent fruits connate only at upper parts and the fused tissues break apart when the infructescences are fully mature.	Adjacent fruits completely free from each other
Fruit type and dehiscence	Capsules dehisced loculicidally and septicidally into four parts from base to apex	Capsules with apical, small perforations	Capsules dehisced loculicidally and septicidally into four parts from base to apex
Seeds	Ellipsoid, bilaterally compressed, shortly winged at both ends	Fusiform, with a long-ventral tail over five times the length of the seed	Ellipsoid, bilaterally compressed, shortly winged at both ends

northern Australia (Table 2). Exceptionally, few myrmecophytic rubiaceous species are known in the Neotropics despite its remarkably high species richness in both Rubiaceae (Andersson, 1992) and myrmecophytes for other plant families (e.g., *Cecropia*, Cecropeciaceae, with 50–60 species; *Tachigali*, Fabaceae, with 20 species; *Triplaris*, Polygonaceae, with 17 species; *Clidemia*, Melastomataceae, with 15–20 species, *Maieta*, Melastomataceae, with 15 species, and *Tococa*, Melastomataceae, with 40–45 species; Davidson and McKey, 1993).

Myrmecophytic plants generally have special structures, commonly known as domatia (sensu Lundström, 1887), that provide nesting space for their ant partners. In Rubiaceae, some myrmecophytes (*Anthorrhiza*, *Hydnophytum*, *Myrmecodia*, *Myrmephytum*, and *Squamellaria*; Huxley and Jebb, 1991a,b,c) have conspicuous, inflated hypocotyls (called chambered tubers by Huxley, 1978). The bases of leaf blades are transformed into vesicle-like structures in a very limited number of species (e.g., *Duroia saccifera*, *Ixora hyppoperifera*, *Remijia*

Table 2
List of Rubiaceae genera and their number of myrmecophytic species and geographic distributions[b]

Genera	Number of the myrmecophyes/ total number of species	Tribes	Subfamilies	Geographic distributions of the myrmecophytes	References
Anthorrhiza	8/9	Psychotrieae	Rubioideae	Papua New Guinea	Huxley and Jebb (1991b), Jebb (1993)
Canthium	4–7+/50[a]	Vanguerieae	Ixoroideae s.l.	Mainland Africa, northern Australia, Papua New Guinea	McKey and Davidson (1993), Gullan and Stewart (1996)
Cuviera	8+/20	Vanguerieae	Ixoroideae s.l.	Mainland Africa	McKey and Davidson (1993)
Duroia	2–3/20[a]	Gardenieae	Ixoroideae s.l.	Neotropics	McKey and Davidson (1993)
Heinsia	1/5	Mussaendeae	Ixoroideae s.l.	Mainland Africa	Robbrecht (1988)
Hoffmannia	1/45[a]	Chioccoceae	Cinchonoideae s.s.	Neotropics	Benson (1985)
Hydnophytum	45/90	Psychotrieae	Rubioideae	Southeast Asia, Fiji Islands, northern Australia	Huxley (1978, 1981)
Ixora	1/400[a]	Ixoreae	Ixoroideae s.l.	Mainland Africa	McKey and Davidson (1993)
Leptactina	1/25[a]	Pavetteae	Ixoroideae s.l.	Mainland Africa	Breteler and Nzabi (1995)
Myrmecodia	26/52	Psychotrieae	Rubioideae	Southeast Asia, northern Australia	Huxley (1978, 1981)
Myrmeconauclea	1/3	Naucleeae	Cinchonoideae s.s.	Borneo, Anambas Island (Indonesia)	Ridsdale (1978)
Myrmephytum	6/8	Psychotrieae	Rubioideae	Southeast Asia	Huxley and Jebb (1991c)
Nauclea	1/9	Naucleeae	Cinchonoideae s.s.	Mainland Africa	Ridsdale (1975)
Neonauclea	17/65	Naucleeae	Cinchonoideae s.s.	Borneo, Philippines, Sulawesi, and Sumatra	Ridsdale (1989)
Psychotria	2/800–1500[a]	Psychotrieae	Rubioideae	Mainland Africa, northern Australia	McKey and Davidson (1993)
Remijia	1–3/25[a]	Cinchoneae	Cinchonoideae s.s.	Neotropics	McKey and Davidson (1993)
Rothmannia	2/40[a]	Gardenieae	Ixoroideae s.l.	Mainland Africa	McKey and Davidson (1993)
Sabicea	1/120[a]	Sabiceeae	Ixoroideae s.l.	Neotropics	McKey and Davidson (1993)
Squamellaria	3/3	Psychotrieae	Rubioideae	Fiji Islands	Jebb (1991)
Tricalysia	1/95[a]	Coffeeae	Ixoroideae s.l.	Mainland Africa	Robbrecht (1979)
Uncaria	2/34	Naucleeae	Cinchonoideae s.s.	Mainland Africa, Southeast Asia	McKey and Davidson (1993), Moog et al. (2003)
Vangueriopsis	2+/4[a]	Vanguerieae	Ixoroideae s.l.	Mainland Africa	McKey and Davidson (1993)

[a] Estimates given by Mabberley (1997).
[b] This table lists all known rubiaceous plants reported to have regularly been occupied by ants and also includes those without obvious and with myrmecophytic traits (e.g., swollen internodes, vesicles-like structures, inflated hypocotyls) for hosting ants.

physophora; Robbrecht, 1988). All myrmecophytic *Myrmeconauclea* and *Neonauclea* s.s. possess conspicuous, swollen internodes (hereinafter internode domatia), whereas the African myrmecophytic *Nauclea vanderguchtii* has rather inconspicuous, swollen internodes. The Bornean myrmecophytic *Myrmeconauclea strigosa* has small, naturally hollowed, and self-opening internode domatia that are inhabited by a range of arboricolous generalist ants (Maschwitz et al., 1989). In contrast, the myrmecophytic *Neonauclea* species bear unopened internode domatia that are colonized by specialized ants.

The 48 non-myrmecophytic *Neonauclea* species (Table 2) together have much larger geographic ranges in comparison with the 17 myrmecophytes (Fig. 1). Of these 48 non-myrmecophytes 41 are regional endemics and distributed as follows: 16 species on New Guinea, eight on the Philippines, six on the Moluccas, four in continental Asia (from India to Vietnam, and Thai-Malay Peninsula), three on Borneo, two on Sulawesi, and one each on Andaman Island (India) and Sumatra. Seven *Neonauclea* species (*N. calycina*, *N. excelsa*, *N. forsteri*, *N. glabra*, *N. lanceolata*, *N. solomonensis*, and *N. truncata*) have wide geographic distributions (Ridsdale, 1989). Of the 17 myrmecophytes eight are restricted to Borneo, six to Sulawesi, two to Sumatra, and only one on both Mindanao and Viscayas Islands (Philippines) (Ridsdale, 1989; Fig. 1). Maschwitz and Fiala (1995) postulated that such more restricted distribution patterns could be taken as indication of a monophyletic origin of the ant–*Neonauclea* associations. These myrmecophytic *Neonauclea* species are restricted to the Malesian region with aseasonal climates, whereas the non-myrmecophytic *Neonauclea* species occur on areas with both aseasonal and seasonal climates throughout the range of the genus (Fig. 1). In addition, the endemic Bornean, Sulawesian, and Sumatran myrmecophytic *Neonauclea* species, respectively, are inhabited by distantly related ant species. At least three formicine *Cladomyrma* species colonize the Bornean myrmecophytic *Neonauclea* (Agosti et al., 1999). The ant genus

Cladomyrma as a whole is absent on Sulawesi and the Philippines. Two *Cladomyrma* species (*C. maschwitzi* and *C. crypteroniae*) shared between Borneo and a restricted area of Central Sumatra (Jambi Province) are host-specific and never colonize either the other myrmecophytic *Neonauclea* species of Borneo or those of Sumatra (Moog and Maschwitz, unpubl. data). In contrast, the myrmicine *Crematogaster* subgenus *Physocrema* species inhabit the Sumatran *Neonauclea* species and that of *Crematogaster* subgenus *Decacrema* species colonize the Sulawesian *Neonauclea* ant–plants (Maschwitz and Fiala, 1995). The structures of the swollen internodes are different in *Neonauclea* species inhabited by *Cladomyrma* and those colonized by *Crematogaster* species. In the former, they are formed by an internode expansion containing soft pith that has to be excavated by the ants. In the latter, however, the internode expansion becomes hollow as a result of the disintegration of the pith prior to ant colonization (Agosti et al., 1999). In both cases, the ant partners have to chew entrance holes into the swollen internodes. These two highly specific morphologies of the swollen internodes of the Bornean and Sulawesian–Sumatran myrmecophytic *Neonauclea*, respectively, might indicate monophyletic origins of these structures, which have independently been exploited by the different ant taxa. On the other hand, multiple origins of myrmecophytism in *Neonauclea* s.s. are also a possibility (e.g., Blattner et al., 2001; Davies et al., 2001; Michelangeli, 2000). None of these hypotheses of the evolution of myrmecophytism in *Neonauclea* s.s. were tested before using molecular-based phylogenies. Therefore, resolving the phylogenetic relationships within the *Ludekia–Myrmeconauclea–Neonauclea* complex was of interest because it provided an opportunity to study the evolution of myrmecophytism in both *Myrmeconauclea* and *Neonauclea* s.s.

In the present study, we use both ETS and ITS sequence data to reconstruct phylogenies for *Neonauclea* s.s. and its allied genera. The resulting phylogenies will subsequently be used as a framework to: (1) re-assess the paraphyly of *Neonauclea* s.s. shown by Razafimandimbison and Bremer (2001, 2002); (2) assess the phylogenetic relationships within the *Ludekia–Myrmeconauclea–Neonauclea* complex; and (3) study the evolution of myrmecophytism within the complex.

2. Materials and methods

2.1. Taxon sampling

We investigated a total of 53 accessions that represented one of the two species of *Ludekia*, the monotypic *Metadina trichotoma*, two of the three species of *Myrmeconauclea*, 16–18 species of *Neonauclea* s.s. (all currently placed in Adininae sensu Razafimandimbison and Bremer, 2002), 14 taxa from the five other subtribes of Naucleeae s.l., and two outgroup taxa, *Cinchona pubescens* and *Exostema lineatum* (see Table 3). The choice of outgroup taxa was in agreement with Razafimandimbison and Bremer (2001, 2002). The accessions of the unidentified myrmecophytic *Neonauclea* (all *Neonauclea* spp. and *Neonauclea* sp. A–E, see Table 3) were collections of young sterile specimens from Borneo. As the determination keys for *Neonauclea* species (Ridsdale, 1989) are primarily based on floral characters, we were unable to identify these collections. It is likely that some of these sterile specimens are new species or identical to some of the known myrmecophytic *Neonauclea* species (Ridsdale, 1989). We kept the informal species names, A–E, as they were already used in Agosti et al. (1999). We excluded from our analyses *Neonauclea brassii* used in Razafimandimbison and Bremer (2002) because its voucher specimen could not be traced to confirm its identity.

2.2. DNA extraction, amplification, and sequencing

The total DNA, extracted from leaves dried in silica gel (Chase and Hills, 1991) and/or herbarium material, was isolated following the mini-prep procedure of Saghai-Maroof et al. (1984), as modified by Doyle and Doyle (1987). We were unable to obtain DNA from herbarium specimens of several non-myrmecophytic *Neonauclea* species. As a result, only five of the 48 described species were included in this study (Table 3).

We repeatedly failed to obtain amplification from the entire intergenic spacer (IGS = NTS + ETS) with the universal primer pair 26-IGS/18S-IGS used by Baldwin and Markos (1998). However, we successfully amplified and sequenced the ETS of all investigated taxa with two primers, 18S-E (5′-GCAGGATCAACCAGGTGACA-3′), designed by Baldwin and Markos (1998) and situated at the 5′ border of 18S and ETS, and ETS-Erit (5′-CTT GTATGGGTTGGTTGGA- 3′), designed by Negrón-Ortiz and Watson (2002) and located at the 3′ end of ETS. For all 50-μL PCRs we added 27.25 μL of H_2O, 5 μL of PCR buffer, 5 μL of $MgCl_2$, 4 μL of DNTP, 0.25 μL of Taq polymerase, 1.25 μL of each primer, and 5 μL of TMACl. PCR amplifications, performed in an Eppendorf Mastercycle gradient (Eppendorf), started with an initial melting phase of 1 min at 97 °C, followed by 40 cycles of 10 s at 97 °C, 30 s at 55 °C, and 20 s at 72 °C and ended with a final extension phase of 7 min at 72 °C.

The entire ITS region (including the 5.8S gene) of all newly investigated *Myrmeconauclea* and *Neonauclea* was amplified with the primer pair P17 (5′-CTACCGAT TGAATGGTCCGGTGAA-3′)/26S-82R (5′-TCCCGG TTCGCTCGCCGTTACTA-3′) (Popp and Oxelman, 2001). These primers attach outside of the 5′ end of ITS1 and 3′ end of ITS2, respectively. We added 0.5 μL of 1%

Table 3
Taxa of Naucleeae s.l. and outgroups used in this study, number of identified paralogues, geographic origins, references, and accession numbers

Taxa	Paralogues	Geographic origins	Voucher information[d] and references	Accession Nos.	
				ITS	ETS
Breonadia salicina			Razafimandimbison and Bremer (2002)	AJ346857	AJ812070
Breonia (as *Neobreonia*) *decaryana*			Razafimandimbison and Bremer (2002)	AJ346859	AJ812072
Cephalanthus occidentalis			Razafimandimbison and Bremer (2002)	AJ346883	AJ812074
Cephalanthus salicifolius			Razafimandimbison and Bremer (2002)	AJ346886	AJ812075
Janotia macrostipula			Razafimandimbison and Bremer (2002)	AJ346869	AJ812071
Ludekia borneensis			Razafimandimbison and Bremer (2002)	AJ346870	AJ812069
Metadina trichotoma			Razafimandimbison and Bremer (2002)	AJ346871	AJ812077
Mitragyna diversifolia			Razafimandimbison and Bremer (2002)	AJ346872	AJ812079
Mitragyna (as *Hallea*) *stipulosa*			Razafimandimbison and Bremer (2002)	AJ346868	AJ812073
Myrmeconauclea stipulacea 1		Borneo (Sabah, Malaysia)	AMO-079, J. Moog (L)	AJ821879	AJ812065
Myrmeconauclea stipulacea 2		Borneo (Sabah, Malaysia)	Beaman 9770 (NY)	AJ821880	AJ812066
Myrmeconauclea strigosa 1			Razafimandimbison and Bremer (2002)	AJ346875	AJ812068
Myrmeconauclea strigosa 2		Borneo (Sabah, Malaysia)	AMO-018, J. Moog (L)	AJ821881	AJ812067
Nauclea vanderguchtii		Gabon	Razafimandimbison 296 (MO, P, TAN)		AJ812083
Neolamarckia cadamba			Razafimandimbison and Bremer (2002)	AJ346878	AJ812082
Neonauclea celebica		Sulawesi (Indonesia)	AMO-128, U. Maschwitz (L)	AJ821870	AJ812051
Neonauclea chalmersii[c]		New Guinea	Brass 28892 (S)	AJ821856	AJ812046
Neonauclea clemensii[c]			Razafimandimbison and Bremer (2002)	AJ346898	AJ812034
Neonauclea cyrtopoda		Sumatra (Indonesia)	AMO-101, J. Moog (L)	AJ821869	AJ812058
Neonauclea forsteri[c]			Razafimandimbison and Bremer (2002)	AJ346880	AJ812033
Neonauclea gigantea		Borneo (Sabah, Malaysia)	AMO-030, J. Moog (L)	AJ821867	AJ812048
Neonauclea glabra[c]		New Guinea	Brass 25477 (S)	AJ821863	AJ812045
Neonauclea longipedunculata[a] (*N. longipedunculata*)			Razafimandimbison and Bremer (2002)		
	ETS_NL1(5)[b]				AJ812052
	ETS_NL2(5)[b]				AJ812054
	ETS_NL3				AJ812053
	ETS_NL4				AJ812055
	ITS_NL1ψ (5)[b]				AJ821882
	ITS_NL2ψ				AJ821883
	ITS_NL3ψ				AJ821884
Neonauclea longipedunculata 1		Borneo (Sabah, Malaysia)	AMO-065, J. Moog (L)	AJ821871	AJ812035
Neonauclea media[c]		Philippines	Elmer 15442 (S)	AJ821864	AJ812044
Neonauclea paracyrtopoda		Borneo (Sarawak, Malaysia)	AMO-071, J. Moog (L)	AJ821865	AJ812047
Neonauclea pseudocalycina		Borneo (Sabah, Malaysia)	AMO-067, J. Moog (L)	AJ821855	AJ812038
Neonauclea sp. 1		Borneo (Sarawak, Malaysia)	AMO-114, J. Moog (L)	AJ821859	AJ812040
Neonauclea sp. 2		Borneo (Sarawak, Malaysia)	AMO-118, J. Moog (L)	AJ821861	AJ812042
Neonauclea sp. 3		Borneo (Sabah, Malaysia)	AMO-119, J. Moog (L)	AJ821877	AJ812063
Neonauclea sp. 4		Borneo (Sabah, Malaysia)	AMO-087, J. Moog (L)	AJ821853	AJ812056
Neonauclea sp. 5		Borneo (Sarawak, Malaysia)	AMO-098, J. Moog (L)	AJ821874	AJ812060
Neonauclea sp. 6		Borneo (Sabah, Malaysia)	AMO-127, J. Moog (L)	AJ821876	AJ812062

(*continued on next page*)

Table 3 (*continued*)

Taxa	Paralogues	Geographic origins	Voucher information[d] and references	Accession Nos.	
				ITS	ETS
Neonauclea sp. 7		Borneo (Sabah, Malaysia)	AMO-124, J. Moog (L)	AJ821862	AJ812043
Neonauclea sp. 8		Borneo (Sarawak, Malaysia)	AMO-042, J. Moog (L)	AJ821857	AJ812037
Neonauclea sp. 9		Borneo (Sarawak, Malaysia)	AMO-112, J. Moog (L)	AJ821860	AJ812041
Neonauclea sp. 10		Borneo (Sabah, Malaysia)	AMO-088, J. Moog (L)	AJ821858	AJ812039
Neonauclea sp. 11		Borneo (Sarawak, Malaysia)	AMO-043, J. Moog (L)	AJ821866	AJ812049
Neonauclea sp. A		Borneo (Sabah, Malaysia)	AMO-035, J. Moog (L)	AJ821852	AJ812036
Neonauclea sp. B		Borneo (Sabah, Malaysia)	AMO-034, J. Moog (L)	AJ821854	
Neonauclea sp. C1		Borneo (Sabah, Malaysia)	AMO-066, J. Moog (L)	AJ821872	AJ812057
Neonauclea sp. C2		Borneo (Sabah, Malaysia)	AMO-080, J. Moog (L)	AJ821873	AJ812059
Neonauclea sp. D1		Borneo (Sabah, Malaysia)	AMO-015, J. Moog (L)	AJ821868	AJ812050
Neonauclea sp. D2		Borneo (Sabah, Malaysia)	AMO-085, J. Moog (L)	AJ821878	AJ812064
Neonauclea sp. E		Borneo (Sarawak, Malaysia)	AMO-074, J. Moog (L)	AJ821875	AJ812061
Pausinystalia macroceras			Razafimandimbison and Bremer (2002)	AJ346890	AJ812076
Pseudocinchona mayumbensis			Razafimandimbison and Bremer (2002)	AJ346864	AJ812078
Uncaria guianensis			Razafimandimbison and Bremer (2002)	AJ414546	AJ812081
Uncaria rhynchophylla			Razafimandimbison and Bremer (2002)	AJ346900	AJ812080
Outgroup taxa					
Cinchona pubescens			Andreasen, Baldwin, and Bremer (1999)	AJ224838	AJ812031
Exostema lineatum			Razafimandimbison and Bremer (2002)	AJ346902	AJ812032

ψ denotes putative pseudogenes.

[a] Polymorphic species.

[b] Number of identical paralogues sampled.

[c] The sampled non-myrmecophytic *Neonauclea* species.

[d] Voucher information is given for new sequences.

of bovine serum albumin (BSA) and 5 μL of 0.1 M tetramethylammonium chloride (TMACl) together in 50 μL PCRs. PCR amplifications, performed in a MJ Research machine (Peltier Thermal Cycler) and/or the same Eppendorf Mastercycle gradient, began with an initial melting phase of 1 min at 97 °C, followed by 35 cycles of 1 min at 95 °C, 1 min 30 s at 55 °C, and 1 min 30 s at 72 °C and ended with a final extension phase of 7 min at 72 °C (hereinafter as 97 °C/rRNA primers). Sequencing reactions were prepared using the two external primers (P17 and 26S-82R) and two internal primers, P16 (5′-TCACTGAACCTTATCATTTAGAGGA-3′) (Popp and Oxelman, 2001) and P25 (5′-GGGTAGTCCCGC CTGACCTG-3′) (Lidén et al., 1995), to produce complete sequences of the entire regions of ITS, with at least partial sequence overlap. The published ITS sequences of the other Naucleeae taxa and *N. longipedunculata* used in this study were amplified and sequenced according to the protocol described in Razafimandimbison and Bremer (2001).

We amplified both the ETS and ITS of the same DNA template of *N. longipedunculata* used in Razafimandimbison and Bremer (2001, 2002) using the 97 °C/rRNA primers. Direct sequencing of its purified PCR products consistently produced multiple sequence signals for both markers, indicating the presence of intraindividual polymorphism. As a result, the PCR products of both *N. longipedunculata* and the other investigated individual (hereinafter *N. longipedunculata* 1) were cloned according to the TOPO TA cloning kit (Invitrogen) described in Razafimandimbison et al. (2004). For both sampled individuals 12 and five white colonies from the ETS and ITS cloning reactions, respectively, were screened and amplified with two universal primers, T7 (5′-AATACGCTCACTATAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′), that were included in the TOPO TA cloning kit. Their respective purified PCR products were sequenced with the 18S-E/ETS-Erit. and P17/26-82R.

In all PCRs, one reaction was run with water instead of DNA as a negative control to check for contamination. All sequencing reactions for both markers were performed with DYEnanicTM ET termination cycle sequencing premix kit (Amersham–Pharmacia Biotech) and subsequently analyzed on a MegaBACE 1000 capillary machine (Amersham–Pharmacia Biotech).

2.3. Data analyses

We performed BLAST searches using the 12 and five new ETS and ITS clonal sequences of *N. longipedunculata*, respectively, to determine whether these sequences were from known fungal endophytes or other contaminants. The percent of their GC contents was computed and the HYPERMUT program package (Rose and Korber, 2000) was used to document the patterns of nucleotide substitution within the 5.8S gene for all identified ITS paralogues of *N. longipedunculata* relative to a reference sequence, *Cephalanthus salicifolia* [see Razafimandimbison et al. (2004) for more information on HYPERMUT]. Sequence data were aligned using CLUSTAL X (Thompson et al., 1997) to produce an initial alignment. This was followed by manual alignment using Se-Al (Rambaut, 1996).

2.3.1. Phylogenetic analyses

We initially performed a parsimony analysis of 53 ETS sequences. Based on the results of this analysis, we selected closer outgroup taxa from within Naucleeae s.l. (*Cephalanthus salicifolia* and *Mitragyna diversifolia*) and subsequently carried out parsimony analyses of both the ITS and combined ETS-ITS sequence data of *Neonauclea* s.s. and its allied genera (*Ludekia*, *Metadina*, and *Myrmeconauclea*). Identical paralogous sequences of *N. longipedunculata* were represented by single sequences in all analyses. We tested for incongruencies between the ETS and ITS matrices with the ILD test (Farris et al., 1995) using the following settings: 1000 replicates, MULPARS option off, and 10 TBR replicates. All separate and combined parsimony analyses were performed with PAUP* version 4.0b (Swofford, 2002) using heuristic searches, with tree bisection-reconnection (TBR) branch swapping, MULPARS option on, 5000 (1000 for the ETS dataset) random sequence additions, and unordered and equally weighted characters. Indels were treated as missing data and all potentially phylogenetically informative indels were rescored as binary (0 and 1) characters. The consistency index (CI; Kluge and Farris, 1969), with uninformative characters excluded, and retention index (RI; Farris, 1989) were calculated. Parsimony jackknife analyses (Farris et al., 1996) of each dataset were performed with PAUP* using heuristic searches, 10000 replicates, percentage of characters deleted in each replicate = 37, MULPARS option off, TBR branch-swapping, emulate option on, 'Jac' resampling method, and five random sequence additions to assess the support of retained clades.

2.3.2. Testing alternative hypotheses of the evolution of myrmecophytism in Neonauclea s.s.

We performed a Bayesian phylogenetic analysis of the combined ETS-ITS dataset (all coded indels excluded) with the computer program MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001) using the GTR + Γ model that was selected by MrModeltest (Nylander, 2001) as the best substitution model, three million Markov chain Monte Carlo (MCMC) generations, with a random starting tree, and sampling every 1000 trees. The temperature of the chains and other parameters were left at default values. After the end of the run, we determined and compared the burn-in based on a graph with generation on the *x*-axis versus log probability of observing the

data on the *y*-axis. We considered the tree with the highest posterior probabilities to be the optimal Bayesian topology for the combined dataset. We subsequently used the one-tailed Shimodaira–Hasegawa non-parametric tests (SH tests: Shimodaira and Hasegawa, 1999; Goldman et al., 2000) to compare statistically the hypothesis of the evolution of ant–*Neonauclea* systems inferred from the optimal (unconstrained) Bayesian topology against three alternative phylogenetic hypotheses: (1) a single origin of the Bornean, Sulawesian, and Sumatran myrmecophytic *Neonauclea* species (Maschwitz and Fiala, 1995); (2) a single origin of the Bornean *Cladomyrma*-inhabited *Neonauclea* species; and (3) a single origin of the non-myrmecophytic *Neonauclea* species. The SH tests were conducted with PAUP* using 1000 bootstrap replicates and resampling estimated by log-likelihood (RELL) optimization.

3. Results

3.1. Amplification and cloning

Direct sequencing of the purified PCR products of all newly investigated *Myrmeconauclea* and *Neonauclea* s.s. and the other sampled Naucleeae taxa yielded single and unambiguous ETS and ITS sequences. The 12 ETS and five ITS clonal sequences of *N. longipedunculata* 1 were identical to those obtained from direct sequencing. In constrast, we obtained 12 ETS (ETS_NL1-4) and five ITS (ITS_NL1-2) divergent paralogous sequences of *N. longipedunculata*. Both ETS_NL1 and ETS_NL2 were recovered five times and ITS_NL1 four times (Table 3). BLAST searches revealed that ITS_NL1 was identical to the published ITS clonal sequence of *N. longipedunculata* used in Razafimandimbison and Bremer (2001, 2002). Therefore, the possibility that these clonal sequences are contaminants can be dismissed.

3.2. Sequence characteristics

The GC contents of the ETS sequences of the sampled *Neonauclea* ranged from 61.35 to 62.50%. The range varied from 61.35 to 62.11% for the four divergent ETS paralogues of *N. longipedunculata* (ETS_NL1-4). The pairwise sequence divergence ranged from 0 to 22.07% for the entire ETS matrix, 0.47–2.20%, and 0–2.20% for the sampled non- and myrmecophytic *Neonauclea* sequences, respectively, and 0–1.70% for the four sampled *Myrmeconauclea* sequences. Finally, the range varied from 0 to 2.55% for the divergent ETS paralogues of *N. longipedunculata*.

The number of nucleotide substitutions within the 5.8S gene of the ITS paralogues of *N. longipedunculata* ranged from 8 to 10. Two of the investigated *Neonauclea* (*Neonauclea* sp. 1 and *Neonauclea* sp. C1) had a single mutation and the remaining *Neonauclea* sequences had no mutation within the 5.8S region. We consider these ITS paralogues of *N. longipedunculata* to represent putative pseudogenes (ψ) and the other sampled *Neonauclea* sequences functional alleles. The same criterion, in combination with estimation of secondary structure stability, was also shown to be powerful for distinguishing the putative pseudogenes from presumed functional sequences of three Naucleeae taxa, *Adinauclea fagifolia*, *Haldina cordifolia*, and *Mitragyna rubrostipulata* in Razafimandimbison et al. (2004). The GC contents of the ITS paralogous sequences of *N. longipedunculata* (ITS_NL1-3) ranged from 57.60 to 57.88% compared to those of the other sampled *Neonauclea* sequences varying from 64.62 to 65.29%. This lower GC content corresponded to the higher frequency of substitution to A and T and less stable secondary structures (higher free energies) (see also Razafimandimbison et al., 2004). The pairwise divergences ranged from 0 to 1.90% between the sampled *Neonauclea* sequences and 0–1.70% between the sampled four *Myrmeconauclea* sequences. They varied from 0.64 to 1.60% and 0–1.90% for the sampled non- and myrmecophytic *Neonauclea* sequences, respectively, and from 0.015 to 14.50% for the sampled ITS paralogues of *N. longipedunculata*.

3.3. Phylogenetic analyses

The alignment of the 53 ETS sequences consisted of 479 base pairs (bp) and contained 161 (33.61%) parsimony-informative characters, of these 7 (4.96%) were indels and 154 (95.04%) were nucleotide substitutions. A parsimony analysis of the ETS data resulted in 36912 most-parsimonious trees (L = 476, CI = 0.616, and RI = 0.772). In the strict consensus ETS tree shown in Fig. 2, both *Myrmeconauclea* and *Neonauclea* s.s. were resolved (JK = 98 and JK = 69, respectively) as monophyletic groups and sister genera (JK = 100). *Ludekia borneensis* was resolved with high support (JK = 94) as sister to the *Myrmeconauclea–Neonauclea* clade. The resolution within both *Myrmeconauclea* and *Neonauclea* s.s. was relatively low. The four clonal ETS paralogues of *N. longipedunculata* did not form a monophyletic group. Instead, they formed two separate subclades: one poorly supported (JK = 58), containing ETS_NL1 and ETS_NL2, and nested in Clade D and the other moderately supported (JK = 84), consisting of ETS_NL3 and ETS_NL4, and embedded in Clade F (Fig. 2). The monophyly of Mitragyninae, Cephalanthinae, Uncarinae, Naucleinae, Breoniinae, and Corynantheinae, all sensu Razafimandimbison and Bremer (2002), was further supported.

Based on the results from the ETS analysis, we selected *Cephalanthus salicifolius* and *Mitragyna diversifolia* as new outgroup taxa from within Naucleeae s.l. to root both the ITS and combined ETS-ITS analyses of

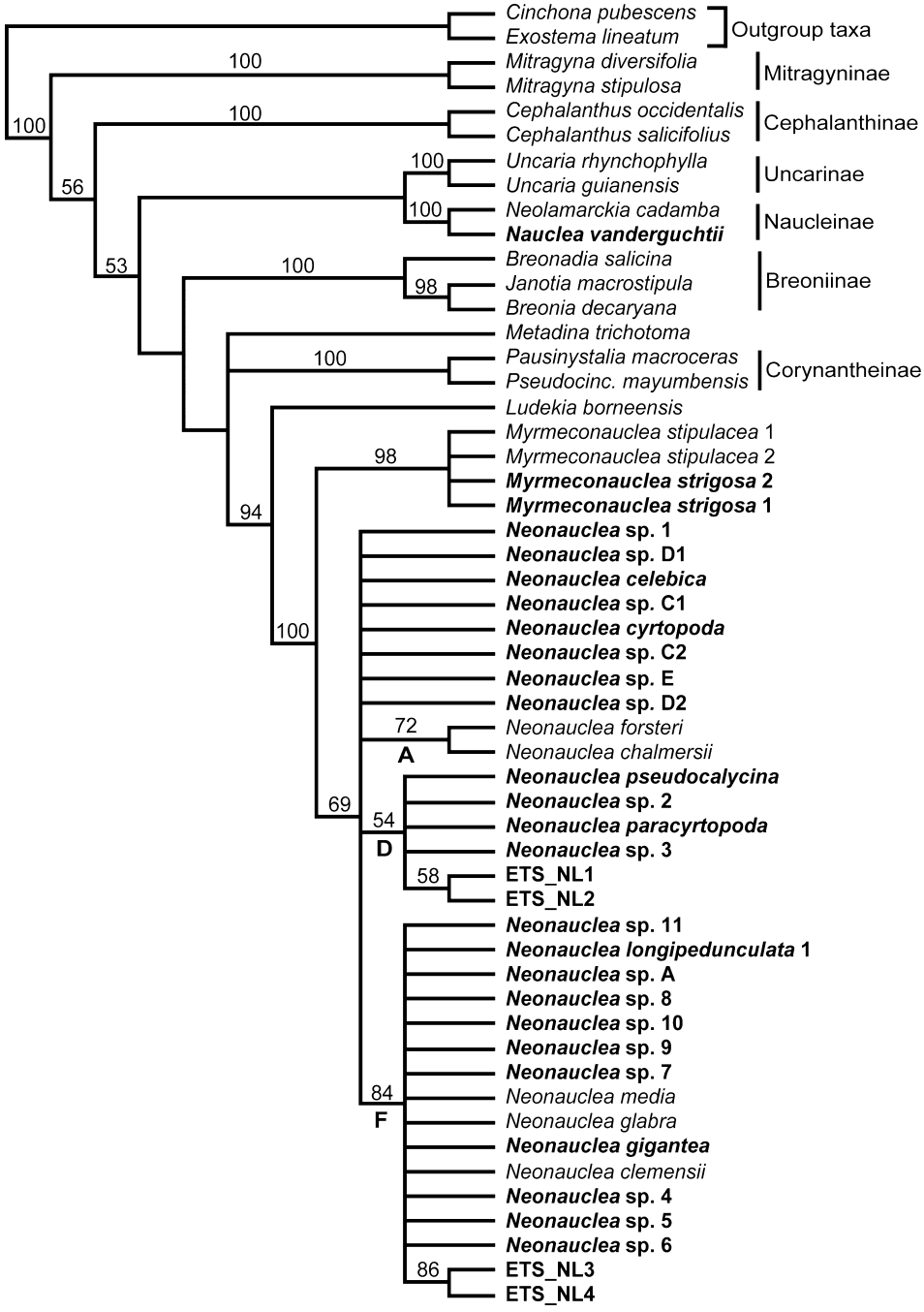

Fig. 2. Strict consensus tree of 36912 most parsimonious trees (L = 476, CI = 0.616, and RI = 0.772) generated from the ETS dataset. Numbers above nodes are jackknife support; capital letters below the nodes are informal names for that node. Taxa in boldface are myrmecophytes. The bracket limits the outgroup taxa and the vertical lines delimit the subtribes.

Neonauclea s.s. and its allied genera. The alignment of 37 ITS sequences consisted of 671 bp and had 58 (8.64%) parsimony-informative characters, of which four (6.89%) were indels and 54 (93.10%) were nucleotide substitutions. A parsimony analysis of the ITS data resulted in 25 most-parsimonious trees (L = 109, CI = 0.679, and RI = 0.778) and the ITS strict consensus tree (all ITS paralogues excluded) is shown in Fig. 3. When the three ITS paralogues of *N. longipedunculata* (ITS_NL1-3) were included, the aligned matrix was 673 bp and had 110 (16.30%) parsimony-informative characters, of which eight (7.28%) were indels and 102 (92.72%) nucleotide substitutions. A parsimony analysis of this ITS data yielded 24 most parsimonious trees (L = 183, CI = 0.727, and RI = 0.830) and a phylogram of one of these 24 most parsimonious trees is shown in Fig. 4. The overall tree topologies of the strict consensus trees without (Fig. 3) and with (result not shown) the ITS putative

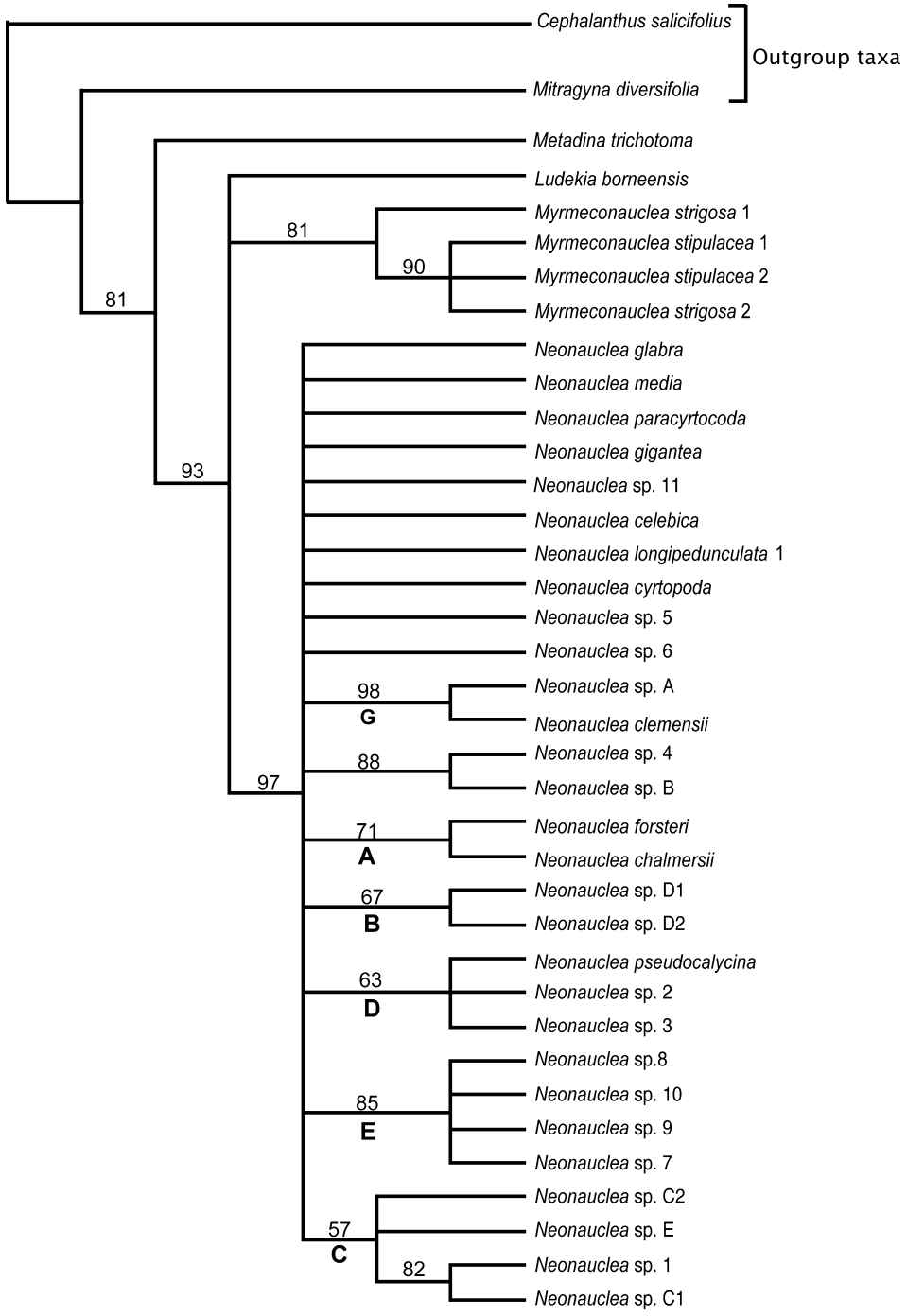

Fig. 3. Strict consensus tree of the 25 most parsimonious trees (L = 109, CI = 0.679, and RI = 0.778) generated from the ITS dataset (the ITS paralogues of NL excluded). Numbers above nodes are jackknife support; capitals letters below the nodes are informal names for that node. The bracket delimits the outgroup taxa.

pseudogenes of *N. longipedunculata* were identical. Like in the phylogram shown in Fig. 4, the three ITS paralogues formed a strongly supported monophyletic group (JK = 100) in the strict consensus tree. This clade was resolved with moderate support (JK = 67) as sister to *Metadina trichotoma*. The most parsimonious trees from a constrained analysis making all sampled *Neonauclea* species (including the three ITS paralogues of *N. longipedunculata*) monophyletic were 11 steps longer than those of the unconstrained analysis. *Ludekia*, *Myrmeconauclea*, and *Neonauclea* s.s. as a group formed a strongly monophyletic group (results not presented).

The results of the partition-homogeneity test indicated that the ETS and ITS datasets were significantly congruent ($P = 0.178$). We then merged the two datasets for 36 taxa in one matrix and performed combined anal-

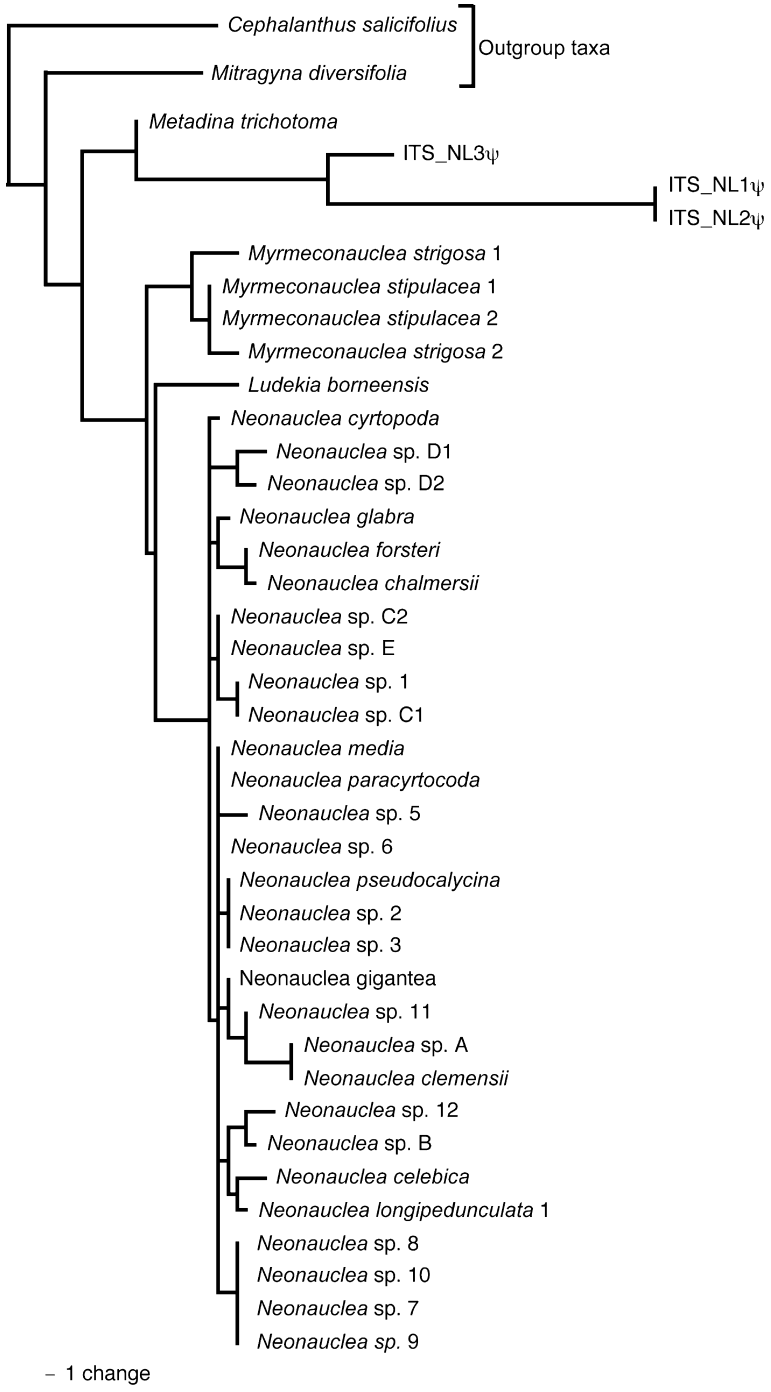

Fig. 4. A phylogram of one of the 24 most parsimonious trees (L = 183, CI = 0.727, and RI = 0.830) generated from the ITS dataset (including the ITS paralogues of NL). The bracket delimits the outgroup taxa. ψ denotes putative pseudogenes.

yses of 1099 bp (430 bp from the ETS matrix and 669 bp from the ITS partition), of which 98 (8.92%) were parsimony-informative characters [43 (43.87%) from the ETS matrix and 55 (56.13%) from the ITS partition]. This yielded 40 most-parsimonious trees (L = 177, CI = 0.650, and RI = 0.799). The strict consensus tree generated from the combined data is shown in Fig. 5 and a phylogram of one of the 40 most parsimonious trees in Fig. 6. *Ludekia*, *Myrmeconauclea*, and *Neonauclea* s.s. as a group formed a strongly monophyletic group (JK = 100). Both *Myrmeconauclea* and *Neonauclea* s.s. were resolved with high support (JK = 100) as sister genera and the resolution within both genera was relatively low (Figs. 5, and 6). Eleven of the 21 Bornean myrmecophytic *Neonauclea* were resolved as more closely related to three of the non-myrmecophytic *Neonauclea* species (*N. clemensii*, *N. glabra*, and *N. media*) than they were to the 10 other Bornean myrmecophytes included in this study.

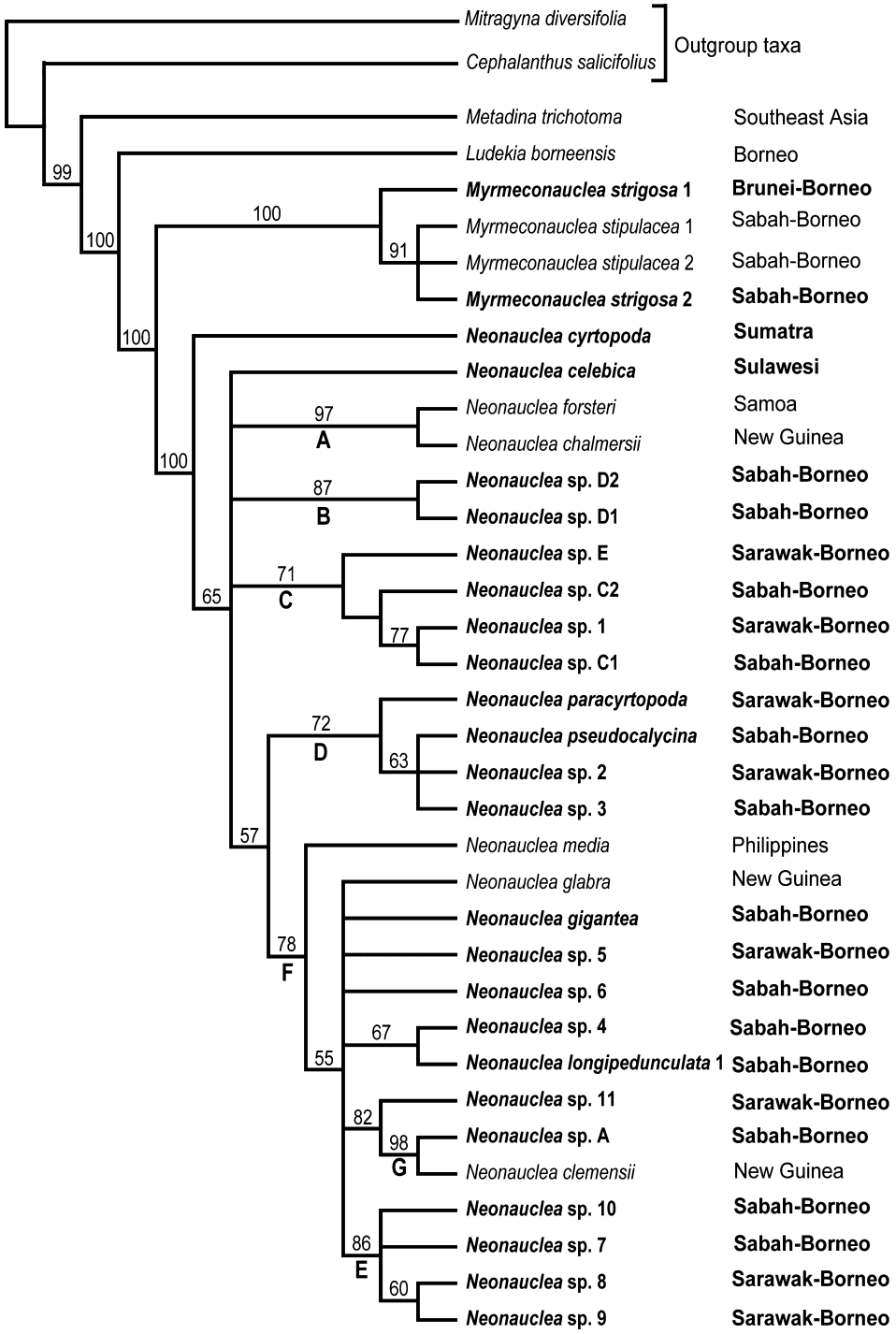

Fig. 5. Strict consensus tree of the 40 most parsimonious trees (L = 177, CI = 0.650, and RI = 0.799) generated from the combined ETS-ITS dataset. Numbers above nodes are jackknife support; capital letters below the nodes are informal names for that node. Taxa in boldface are myrmecophytes. Geographic distributions of the sampled *Ludekia*, *Metadina*, *Myrmeconauclea*, and *Neonauclea* are listed next to the cladogram. The bracket delimits the outgroup taxa.

There were, however, some differences with weak support in resolution between the ETS, ITS, and combined trees (Figs. 2, 3, and 5). For examples, the support of the monophyly of *Neonauclea* s.s. was moderate (JK = 69) in the ETS tree (Fig. 2), whereas this clade received strong support (JK = 97 and 100, respectively) in both the ITS (Fig. 3) and combined (Fig. 5) trees. The support for the sister-group relationships between *Myrmeconauclea* and *Neonauclea* s.s. was very high (JK = 100) for both the ETS and combined trees. This relationship was unresolved in the ITS tree (Fig. 3). The subclades B–C and E and the subclade F that were collapsed in the ETS tree

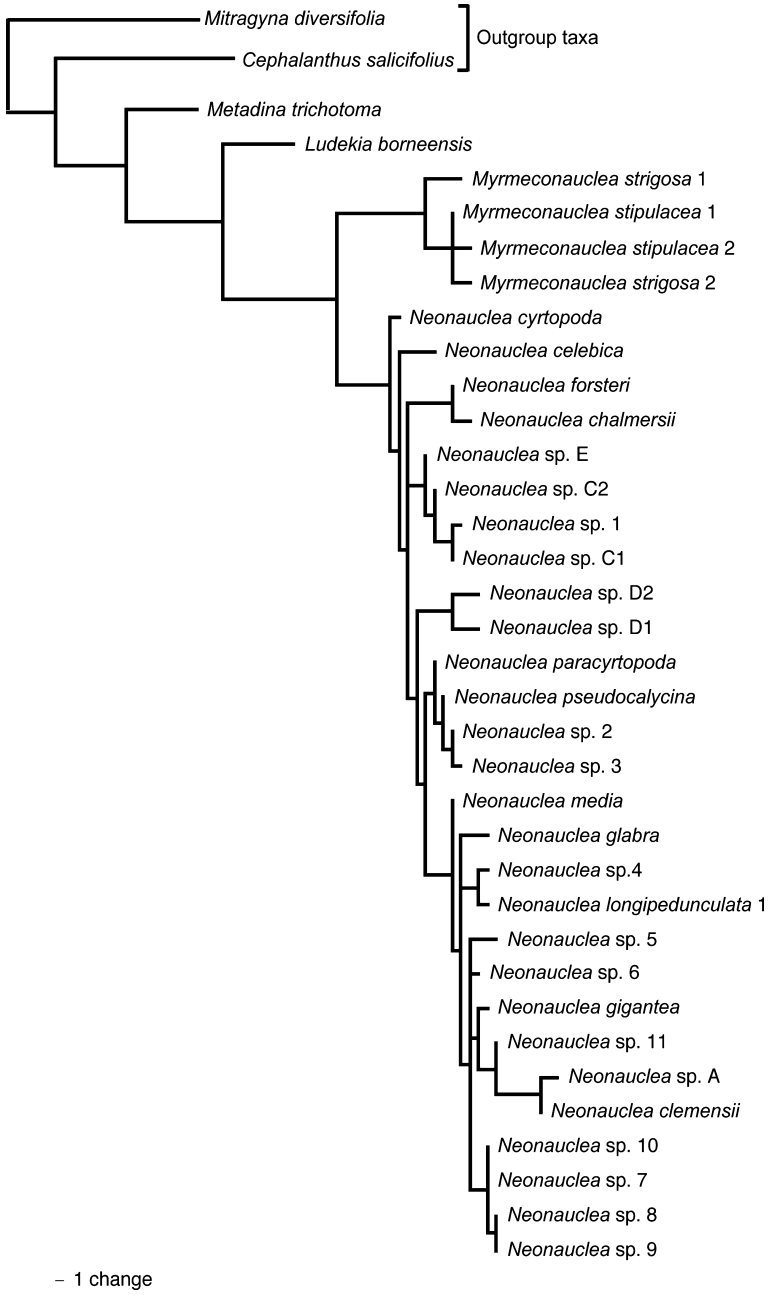

Fig. 6. A phylogram of the 40 most parsimonious trees (L = 177, CI = 0.650, and RI = 0.799) generated from the combined ETS-ITS datasets. The bracket delimits the outgroup taxa.

Table 4
Log likelihood scores for three alternative tree topologies using the Shimodaira–Hasegawa (SH) tests

Hypotheses	Score (−ln L)	Difference (−ln L)	Significance (*P*)	Rejected
The optimal Bayesian tree	3290.08	—	Best	
Single (monophyletic) origin of all sampled myrmecophytic *Neonauclea* species	3374.51	84.43	<0.01*	Yes
Single origin of all Bornean *Cladomyrma*-inhabited *Neonauclea* species	3366.58	76.50	<0.01*	Yes
Single origin of all five non-myrmecophytic *Neonauclea* species	3343.55	53.47	<0.01*	Yes

* $P < 0.05$.

(Fig. 2) and ITS tree (Fig. 3), respectively, were all resolved in the combined tree (Fig. 5). In addition, three non-myrmecophytic species (*Neonauclea clemensii*, *N. glabra*, and *N. media*) together with 13 and 11 myrmecophytic *Neonauclea* accessions formed moderately supported (JK = 84 and 78, respectively) monophyletic groups in the ETS (Fig. 2) and combined (Fig. 5) trees; these relationships were collapsed in the ITS tree (Fig. 3). Finally, the subclade G was strongly supported in both the ITS and combined trees but collapsed in the ETS tree.

The results of SH tests are summarized in Table 4. The tree topologies of the three alternative phylogenetic hypotheses of the evolution of myrmecophytism were significantly different from that of the optimal Bayesian tree and therefore can be rejected as adequate description of the combined dataset.

4. Discussion

4.1. Comparison between the evolution of ETS and ITS in Naucleeae s.l.

Both ETS and ITS (including 5.8S gene) regions are parts of 18S-26S nuclear rDNA and parts of the same transcriptional unit. They have interdependent roles in the maturation of rRNAs and hence, may be evolving under similar functional constraints and at comparable rates (e.g., Musters et al., 1990). However, the higher level of intraindividual polymorphism discovered in the identified ITS paralogues (0.015–14.50%) of *N. longipedunculata*, most likely due to recent pseudogene formation, compared to that found in its sampled ETS paralogous sequences (0–2.55%) seems to indicate that concerted evolution (Zimmer et al., 1980) operates at different rates in the ETS and ITS regions of *N. longipedunculata*. On the other hand, it is possible that our amplifications missed some of the variation in either the ETS or ITS for *N. longipedunculata*; plus, our clone sampling is rather limited for both markers. In addition, the pairwise sequence divergence between ETS_NL1 and ETS_NL2 (0.243%), both nested in Clade D, and between ETS_NL3 and ETS_ NL4 (0.50%), both embedded in Clade F (Fig. 2), are lower than between the ETS_NL1-ETS_NL2 subclade and the ETS_NL3-ETS_NL4 subclade (0–2.55%). This indicates that the homogenization process occurs more quickly within than between the non-homologous chromosomes of *N. longipedunculata.*

When the aligned ETS and ITS matrices used in the combined analyses are compared, the former (with 430 bp) is 239 bp shorter than the latter (with 669 bp). Yet, 42 (9.76%) of the 430 bp are parsimony-informative characters in the ETS partition in comparison with 55 (8.22%) of 669 bp in the ITS matrix. On the other hand, the ETS partition contributes only to 43.87% of the total number of parsimony-informative characters (97) in the combined analyses. Therefore, our studies corroborate the conclusions of Baldwin and Markos (1998) that "the ETS holds great promise for augmenting ITS data for phylogenetic studies of young lineages." In addition, the levels of homoplasy are comparable in the ETS (CI = 0.619) and ITS (CI = 0. 679) data, also in agreement with Baldwin and Markos (1998).

4.2. Monophyly of Myrmeconauclea and Neonauclea s.s.

The ETS, ITS, and combined trees (Figs. 2–6) all support the monophyly of *Myrmeconauclea* (Ridsdale, 1978) and *Neonauclea* s.s. (Ridsdale, 1978, 1989) and their sister-group relationships, consistent with morphological data (Ridsdale, 1978; Table 1). The monophyly of *Ludekia*, *Myrmeconauclea*, and *Neonauclea* s.s. together as a group indicated by Razafimandimbison and Bremer (2002) is further supported by the analyses presented (Figs. 2–6). *Ludekia*, represented here by the type species, *L. borneensis*, is strongly supported to be sister to the *Myrmeconauclea–Neonauclea* clade, also in agreement with morphological data (Table 1). In the light of the evidence presented, we continue to maintain the current generic status of these genera.

4.3. Assessment of causes of the paraphyly of Neonauclea s.s. in Razafimandimbison and Bremer (2001, 2002) and the present ITS analysis

The analyses presented here raise questions as to why *Neonauclea* s.s. was resolved as paraphyletic in Razafimandimbison and Bremer (2001, 2002). Our ETS and ITS analyses suggest different positions for *N. longipedunculata*. The ETS tree (Fig. 2) resolves all four ETS sequences of *N. longipedunculata* within the *Neonauclea* clade. In contrast, the strict consensus tree (not presented) from the ITS analysis with *N. longipedunculata* (depicting almost identical tree topologies as the phylogram in Fig. 4) resolves both *Metadina trichotoma* and *N. longipedunculata* as sister taxa, consistent with Razafimandimbison and Bremer (2001). Using both the "Trace All Changes" and "Trace Labeling" options in MacClade 4.0 (Maddison and Maddison, 2000), we find that two (sites # 96 and # 126) of the three autapomorphic substitutions of *M. trichotoma* and two nucleotide substitutions of the three ITS paralogues of *N. longipedunculata* are coincidently resolved as synapomorphies. The collapse of their sister–taxon relationships after exclusion of these two sites seems to indicate that the paraphyly of *Neonauclea* s.s. in Razafimandimbison and Bremer (2001, 2002) and our ITS analysis (Fig. 4) appears to be the result of parallel substitutions in *M. trichotoma* and the three sampled ITS putative pseudogenes of *N. longipedunculata.*

When the same ITS dataset is analyzed (results not shown) with MrBayes (Huelsenbeck and Ronquist, 2001)

using the GTR + Γ substitution model both *Metadina trichotoma* and *N. longipedunculata* are still resolved as sister taxa and the overall tree topologies are almost identical to the parsimony-based tree (Fig. 4). Furthermore, we find that the other sampled ITS *Neonauclea* sequences and the three ITS paralogues of *N. longipedunculata* still do not form a clade even after exclusion of the sites # 96 and # 126; the *N. longipedunculata* clade is left unresolved outside of the *Ludekia–Myrmeconauclea–Neonauclea* clade, indicating that some of the synapomorphic sites of *Neonauclea* s.s. must have randomly been mutated in the three sampled ITS paralogues of *N. longipedunculata*. Accordingly, all autapomorphic substitutions that strongly group the three ITS pseudogenes together appear to have evolved independently .

The analyses presented here provide additional evidence, which can explain why inclusion of the 76 highly divergent ITS putative pseudogenes of *Adinauclea fagifolia*, *Haldina cordifolia*, and *Mitragyna rubrostipulata* (all belonging to Naucleeae s.l.) in both the Bayesian and parsimony analyses in Razafimandimbison et al. (2004) did not lead to spurious relationships. The fact that the five presumed functional sequences and the 26 identified putative pseudogenes of *Adinauclea fagifolia* all form a highly supported (PP = 100, Fig. 5 and JK = 86, Fig. 6, Razafimandimbison et al., 2004) monophyletic group strongly indicates that these pseudogenes still retain some of the synapomorphies (sites # 32, # 37, # 65, # 135, # 200, # 208, # 342, and # 479) of their functional counterparts. Also, the fact that all 28 sampled putative pseudogenes of *Mitragyna rubrostipulata*, when analyzed together with two *Mitragyna* species (*M. diversifolia* and *M. stipulosa*), still form a strongly supported clade provides further support for this argument. These paralogous ITS sequences are united by five synapomorphies (sites # 316, # 409, # 522, # 528, and # 548). Based on the evidence presented, it appears that whether or not inclusion of putative pseudogenes in phylogenetic analyses leads to incorrect phylogenies is dependent upon the balance between the number of synapomorphic sites of their functional counterparts retained and the number of sites coincidently resolved as synapomorphies with the other ingroup taxa.

Finally, we investigate whether exclusion of *N. longipedunculata* affects the phylogenetic relationships and the intratribal classification of Naucleeae proposed in Razafimandimbison and Bremer (2001, 2002). We find that the overall tree topologies of the ITS and combined analyses remain the same (results not shown).

4.4. Causes of incongruence between ETS and ITS trees regarding the placement of N. longipedunculata

We checked the identification of all newly investigated *Myrmeconauclea* and *Neonauclea* species included in this study and confirmed that they were all correctly identified. We argue that the incongruence between the ETS (Fig. 2) and ITS (Fig. 4) trees regarding the placement of *N. longipedunculata* is probably the result of the combined effects of new pseudogene formation and a recent hybridization event or incomplete lineage sorting. Concerted evolution may have a limited genomic scope and rate both within and between rDNA loci because highly divergent ITS paralogues seem to be common in *N. longipedunculata*. We suspect that PCR selection (Wagner et al., 1994) is the reason why we have only sampled the low-GC pseudogenes despite using different PCR conditions (high denaturing temperatures, PCR additives, and primers). Alternatively, the putative ITS pseudogenes could be located at one or more major inactive loci and the functional ITS copies could reside at a minor functional locus; in that case, the number of the pseudogenes would remain higher than that of the functional copies after amplification.

The placement of the ETS clonal sequences of *N. longipedunculata* in two separate clades within *Neonauclea* s.s. (Fig. 2) could be an indication of either a recent hybridization event, involving two *Neonauclea* species, one belonging to Clade D and the other to Clade F, or incomplete lineage sorting. All sampled myrmecophytic *Neonauclea* in both Clades D and F are endemics to Borneo. Hybridization is possible because the sampled *Neonauclea* species from Sabah, Borneo (e.g., *Neonauclea gigantea* and *N. pseudocalycina*) are likely to grow in sympatry with *N. longipedunculata* (collected from the Danum Valley of Sabah). Plus, the absence of intraindividual polymorphism in the ETS and ITS investigations of *N. longipendunculata* 1 (also from Sabah) appears to favor the hybridization hypothesis because the polymorphic allele in question would be expected to be more randomly distributed among individuals under a scenario of incomplete lineage sorting. Unfortunately, the *rbc*L and *trn*T-F sequences of *Ludekia*, *Myrmeconauclea*, and *Neonauclea* s.s. are almost identical (Razafimandimbison and Bremer, 2001, 2002); hence, their resulting phylogenies do not provide any additional evidence regarding the position of the maternal (ovule donor) plant of *N. longipedunculata*.

4.5. Evolution of myrmecophytism within Naucleeae s.l.

The ETS tree (Fig. 2) indicates that myrmecophytism has evolved at least two or three times within Naucleeae sensu Razafimandimbison and Bremer (2002): the African *Nauclea vanderguchtii* (Ridsdale, 1975; Fig. 2), *Myrmeconauclea strigosa* (Ridsdale, 1978; Fig. 2–6), and 17 *Neonauclea* species (Ridsdale, 1989). These myrmecophytic Naucleeae species appear to have independently been exploited by different ant taxa in different geographic areas. *M. strigosa* is colonized by a range of arboricolous ants, whereas the myrmecophytic *Neonauclea* species are inhabited by specialized ants: *Cladomyrma* species on Borneo (Agosti et al., 1999), *Crematogaster*

subgenus *Decacrema* species on Sulawesi, and *Crematogaster* subgenus *Physocrema* species on Sumatra (Maschwitz and Fiala, 1995). According to Bequaert (1922: p. 460), *N. vanderguchtii* (as *Sarcocephalus* sp.) is inhabited by an African *Crematogaster* species.

4.6. Evolution of myrmecophytism within the Myrmeconauclea–Neonauclea clade

We use the phylogenetic tree from the combined analysis (Fig. 5) for basing conclusions on both the evolution of myrmecophytism in *Neonauclea* s.s. and its diversification in Southeast Asia, as it is the best-supported hypothesis, maximizing congruence among all of the characters sampled. Based on the sister-group relationships between *Myrmeconauclea* and *Neonauclea* s.s., we put forward two competing hypotheses regarding the evolution of their myrmecophytism: (H1) both *Myrmeconauclea* and *Neonauclea* s.s. had a myrmecophytic common ancestor. There were subsequent losses of myrmecophytic traits in their respective progenitors before their divergence, followed by independent acquisition of myrmecophytism in the Bornean, Sulawesian, and Sumatran *Neonauclea* species; and (H2) the sister genera had a non-myrmecophytic common ancestor and their Bornean (for both genera) and Sulawesian and Sumatran (for *Neonauclea* s.s.) species subsequently acquired myrmecophytism independently only after their divergence and/or radiations (Fig. 1). The disparities in the nature of the ant–*Myrmeconauclea* (Maschwitz et al., 1989) and ant–*Neonauclea* systems (Maschwitz and Fiala, 1995) seem to provide support for the hypothesis H2. Plus, the hypothesis H1 requires two additional losses of myrmecophytic traits and therefore is not the most parsimonious solution. Our limited sampling of the non-mymecophyte *Neonauclea*, coupled with some *Neonauclea* species with ambiguous positions (Fig. 5) have refrained us from using any likelihood-based approach for estimating the degree of confidence in ancestral character state of the common ancestor (non-myrmecophyte versus myrmecophyte) of *Myrmeconauclea* and *Neonauclea* s.s.

Eleven of the 21-Bornean myrmecophytic *Neonauclea* accessions seem to be more closely related to three of the five non-myrmecophytic *Neonauclea* species (*N. clemensii*, *N. glabra*, and *N. media*) than they are to the other 10-Bornean myrmecophytic *Neonauclea* (Fig. 5). The SH tests (Table 4) indicate that all three alternative (constrained) hypotheses [(1) a single origin of the myrmecophytic *Neonauclea* species (Maschwitz and Fiala, 1995); (2) a single origin of the Bornean *Cladomyrma*-inhabited *Neonauclea* species; and (3) a single origin of the non-myrmecophytic *Neonauclea* species] are significantly different from the optimal (unconstrained) Bayesian hypothesis (at $P < 0.05$ level). Therefore, the analyses presented seem to support multiple origins of the Bornean myrmecophyte *Neonauclea* but perceive no support for either the hypothesis of a single origin of the Bornean myrmecophytic *Neonauclea* species or that of monophyletic origin of the non-myrmecophyte *Neonauclea* (see Table 4). Multiple independent origins of ant–plant associations have also been inferred from phylogenetic studies of other tropical plant genera [e.g., *Macaranga*, Euphorbiaceae (Blattner et al., 2001; Davies et al., 2001; Feldhaar et al., 2003); *Tococa*, Melastomataceae (Michelangeli, 2000)]. According to Agosti et al. (1999), the Bornean myrmecophyte *Neonauclea* appear to have independently been colonized by at least three Bornean *Cladomyrma* ant species, *C. dianeae*, *C. hewiti*, and *C. maryatiae*. *Cladomyrma dianeae* appears to be the most predominant ant species, which is known to colonize at least four described Bornean *Neonauclea* species, *N. borneensis*, *N. gigantea*, *N. longipedunculata*, and *N. paracyrtopoda*, many of our unidentified *Neonauclea* species (Agosti et al., 1999; Moog and Maschwitz, unpubl. data), and probably the other four known myrmecophytic *Neonauclea* from Borneo, *N. artocarpoides*, *N. calcarea*, *N. excelsioides*, and *N. pseudocalycina*. Herbarium specimens of these latter species at the Leiden herbarium (L, Holmgren et al., 1990) show the typical entrance holes of *Cladomyrma* (Moog, pers. obs.). *Cladomyrma maryatiae* inhabits *N. pseudocalycina* and three distinct unidentified *Neonauclea* species; *C. hewiti* colonizes both *N. longipedunculata* and *N. pseudocalycina*. Furthermore, our findings indicate that the excavated internode domatia of the Bornean myrmecophytic *Neonauclea* may not be homologous because these ant–plants appear to be either para- or polyphyletic. In this case, the uniform morphological characters must have evolved through parallel evolution and therefore are likely to mislead morphological-based phylogenies if included in analyses.

The absence of the myrmecophytic *Neonauclea* species outside the range of their ant partners (e.g., continental Asia, Java, the Moluccas, New Guinea) (see Fig. 1) seems to support the conclusions of Blattner et al. (2001) that the establishment of new myrmecophytic populations in new habitats is dependent upon the availability of their specific ant partners. This would also imply that obligate mutualistic ant–plant associations, to some extent, limit vagility of myrmecophytes. However, this pattern could be obtained if the non-myrmecophytic *Neonauclea* species of New Guinea and its neighboring islands had a Bornean non-myrmecophytic *Neonauclea* ancestor(s), that successfully dispersed there before the Bornean *Cladomyrma* species began to colonize the Bornean *Neonauclea* species. Alternatively, our results could also indicate that at least three non-myrmecophytic *Neonauclea* species (*N. clemensii*, *N. forsteri*, and *N. media*) descended from a myrmecophytic *Neonauclea* ancestor(s) if the Bornean *Cladomyrma* species had colonized the Bornean *Neonauclea* species before a

myrmecophytic Bornean ancestor(s) of the non-myrmecophytic *Neonauclea* of New Guinea and its neighboring islands dispersed and radiated there. We are unable to choose between these two competing hypotheses, as the phylogeny of *Cladomyrma* is currently unknown and our sampling of the non-myrmecophytic *Neonauclea* is rather limited.

Davies et al. (2001) argue that seasonality constrains the distribution of the myrmecophyte *Macaranga* in western Malesia due to the discontinuity in food supply (in form of 'food bodies') for their ant partners. However, the absence of the myrmecophytic *Neonauclea* species in other aseasonal areas (e.g., New Guinea and the Moluccas, Fig. 1) cannot directly be explained by resource limitation and/or seasonality because these ant–plants produce only nesting space for their ant partners, which feed mainly on the honeydew of their trophobionts (Maschwitz and Fiala, 1995). On the other hand, it has been shown that resource limitation or seasonality indirectly affect species composition of the trophobiotic mealybugs and the quantity and/or quality of the honeydew produced by the trophobionts (Risebrow and Dixon, 1987).

We are unable to draw any conclusions regarding the evolution of ant–*Neonauclea* associations on Sulawesi and Sumatra, as our studies include only one myrmecophytic *Neonauclea* species from each island (see Table 3). Evidently, more thorough taxon sampling of the non-myrmecophytic *Neonauclea* species is required to further evaluate the hypotheses of the evolution of myrmecophytism in the *Myrmeconauclea–Neonauclea* clade put forward in this study. Plus, ambiguity in placement of some *Neonauclea* species suggests that additional sources of phylogenetically informative characters are needed.

4.7. Rapid radiation in Neonauclea s.s. versus slow radiation in Myrmeconauclea

By definition, two sister groups are of equal age and have equivalent histories up to the time of their divergence. This implies that differences in their species diversities can indicate disparities in their diversification rates (Hodges, 1997). The sister genera *Myrmeconauclea* and *Neonauclea* s.s. have unbalanced species richness (3 and 65 species, respectively). We interpret the low level of variation in both the ETS and ITS sequences as an indicative of a rapid and recent radiation for *Neonauclea* s.s. (e.g., Harris et al., 2000; Malcomber, 2002) but a slow and recent radiation for *Myrmeconauclea*. Both genera belong to the subtribe Adininae sensu Razafimandimbison and Bremer (2002), which also shows a similar pattern of genetic variation (Razafimandimbison and Bremer, 2001, 2002). In addition, the level of variation found in both the ETS and ITS of the other investigated Naucleeae genera are two (e.g., *Mitragyna* and *Uncaria*) or even three (e.g., *Cephalanthus*, *Nauclea*, and *Pausinystalia*) times higher (Razafimandimbison and Bremer, unpubl. data) than those found within *Myrmeconauclea* (0–1.70%) and *Neonauclea* (0–2.20%) species included in this study. In the light of the above evidence, we consider both *Myrmeconauclea* and *Neonauclea* s.s. to be young genera in Naucleeae s.l. We are unable to perform a molecular clock test or estimate diversification rates due to our limited sampling of the non-myrmecophytic *Neonauclea* species. So, a decrease of nucleotide substitution in the ETS and ITS remains to be another explanation for the observed diversification patterns in the two genera.

The differences in both the species diversity and geographical patterns of *Myrmeconauclea* and *Neonauclea* s.s. could partly be explained by disparities in the nature of their fruits and seeds and their ability to colonize a wide range of habitats.

4.7.1. Recent and rapid radiation in Neonauclea s.s.

All members of *Neonauclea* s.s. produce dry, capsular fruits and small, ellipsoid, and bilaterally compressed seeds that are shortly winged at both ends. We argue that these wind-dispersed seeds have allowed *Neonauclea* species to reach new suitable habitats. The occurrence of the endemic *Neonauclea* species on almost all Southeast Asian islands (e.g., 11 species on Borneo, 15 on New Guinea, nine on the Philippines, eight on Sulawesi, six on the Moluccas, two on Sumatra, Ridsdale, 1989) strongly indicates that the diversification of *Neonauclea* s.s. has been associated by limited interisland dispersals and subsequent intraisland radiations. Unlike *Ludekia* and *Myrmeconauclea*, many *Neonauclea* species show considerable ecological amplitude ranging from low, mid-altitude rainforests to dry deciduous or limestone forests, ultra basic soils to swamped or riverine forests (Ridsdale, 1970, 1989). This wide ecological tolerance would preadapt them for colonizing new habitats before speciation. Accordingly, we argue that both geographical and ecological opportunities (e.g., availability of new suitable habitats and lower competition on entry of the new geographical areas) may have partly been responsible for the rapid diversification of the non-myrmecophytic *Neonauclea*. In addition, all other Adininae genera, except *Myrmeconauclea*, produce relatively similar type of capsular fruits and winged seeds as found in *Neonauclea* s.s., indicating that other constraints rather than seed dispersal limit the geographic range of *Ludekia*. Also, this evidence indicates that capsular fruit is an ancestral character retained in *Neonauclea* s.s.

Furthermore, it is unlikely that a shift in pollinator could have triggered the rapid diversification of the non-myrmecophytic *Neonauclea* species, as the color and shape of flowers of both the non- and myrmecophytes are the same and the lengths of their corollas are similar (4–18 mm long in the former and 6–18 mm long in the latter). This indicates that the flowers of *Neonauclea* s.s. may be pollinated by the same range of pollinators.

Maschwitz and Fiala's (1995) study of the Sumatran myrmecophytic species, *Neonauclea cyrtopoda* and the Sulawesian myrmecophytic species, *N. celebica* and Moog's pers. obs. (unpubl. data) on *N. cyrtopoda* and the Bornean myrmecophytic species, *N. gigantea*, all reveal that the flowers of these *Neonauclea* species are visited by several species of butterflies and moths, beetles (chrysomelids and scarabaeids), and thrips. Beetles appear to be the important pollinators, especially in *N. cyrtopoda*. Also, Weber's (1999) study on the non-myrmecophytic species, *Neonauclea pallida*, shows that its flowers are visited by several species of beetles, moths, and stingless bees, but pyralid moths are the most important pollinators.

The derived position of the non-myrmecophytic *Neonauclea clemensii* and *N. glabra* (New Guinea) and *N. media* (Philippines) within the Bornean–Sulawesian myrmecophytic *Neonauclea* clade (Fig. 5) seems to indicate that they could have had a myrmecophytic *Neonauclea* progenitor(s) from either Borneo or Sulawesi. This is under the assumption that their ancestor dispersed to New Guinea and its neighboring islands after *Cladomyrma* and *Crematogaster* subgenus *Decacrema* species, respectively, colonized the Bornean and Sulawesian *Neonauclea* species. We postulate that their ancestors were successfully dispersed outside the range of their ant partners (Fig. 1) and subsequently adapted to the absence of their ant partners by losing their internode domatia. Losses of myrmecophytic traits have also been reported for *Cecropia peltata* when introduced into areas outside its natural geographic range (McKey, 1988; Putz and Holbrook, 1988; Rickson, 1977). On the other hand, the three non-myrmecophytic *Neonauclea* could have had non-myrmecophytic *Neonauclea* ancestors if the colonization of *Cladomyrma* and *Crematogaster* subgenus *Decacrema* species took place only after their ancestors dispersed to New Guinea or its neighboring islands.

The occurrence of some non-myrmecophytic *Neonauclea* species in sympatry with the Bornean and Sulawesian myrmecophytic *Neonauclea* species (e.g., *N. angustifolia*, *N. endertii* on Borneo; *N. intercontinentalis*, *N. pseudopeduncularis* on Sulawesi) could be explained by the re-invasion of their respective non-myrmecophytic progenitors in the aseasonal areas currently occupied by the myrmecophytes, followed by subsequent speciations (see also Blattner et al., 2001; Davies et al., 2001). Alternatively, these non-myrmecophytes could be the Bornean and/or Sulawesian *Neonauclea* species that might never have been colonized by *Cladomyrma* or *Crematogaster* subgenus *Decacrema* species.

The rates of nucleotide substitutions (based on the pairwise sequence divergences) of the ETS data are almost identical for the sampled non- (0.47–2.20%) and myrmecophytic (0–2.20%) *Neonauclea* species. In the ITS data, the rates are also comparable (0.617–1.60% for the former and 0–1.87% for latter), indicating that their rates of diversifications are similar. The non-monophyly of the Bornean myrmecophytic *Neonauclea* and this similarity in the nucleotide substitution rates of the sampled non- and myrmecophytic *Neonauclea* both seem to indicate that the acquisition of myrmecophytism could not have been the key innovation (sensu Heard and Hauser, 1995) responsible for the radiations of the myrmecophyte *Neonauclea*. On the other hand, both our phylogenetic hypothesis (Fig. 5) and estimates of the rates of diversification for *Neonauclea* s.s. are based on limited sampling of the non-myrmecophytic *Neonauclea* species. Therefore, a thorough sampling of both the non- and myrmecophytic *Neonauclea* species is clearly needed to re-evaluate our hypotheses on how the diversification of the non-myrmecophyte *Neonauclea* is related to the evolution of the myrmecophytic ones.

4.7.2. Recent and slow radiation in Myrmeconauclea

The acquisition of the new morphological traits (pseudo-multiple fruits and long-tailed, fusiform seeds) appears to have allowed *Myrmeconauclea* to specialize on rheophytic habitats. Accordingly, the genus has a narrow ecological tolerance that in turn may have hindered its speciation [see also Barrett and Graham (1997); Givnish (1997)]. Merrill (1920, 376) postulated that the dissemination of the mature seeds of *Myrmeconauclea* through the small perforation of the fruits would take a considerable period of time. However, when the mature fruits of *Myrmeconauclea* fall in the rivers or creeks, the penetrating water could enhance the seed release.

In addition, the low species richness in *Myrmeconauclea* might have partly been caused by high intraspecific competition among previously and simultaneously established siblings, high parent-sibling competition, and/or predation from pathogens and predators that may have been accumulated on the parent plants or siblings nearby (Tiffney and Mazer, 1995).

5. Conclusions

In conclusion, the phylogenetic analyses presented all show support for the monophyly of both *Myrmeconauclea* (Ridsdale, 1978) and *Neonauclea* s.s. (Ridsdale, 1989), in agreement with morphological data. The present study further support close relationships between *Ludekia*, *Myrmeconauclea*, and *Neonauclea* s.s. and the sister-group relationships between the two latter genera, suggested by Ridsdale (1978) based on floral morphologies. We demonstrate that the previously proposed paraphyly of *Neonauclea* s.s. in Razafimandimbison and Bremer (2001, 2002) and our ITS analysis (Fig. 4) may be the result of the combined effects of parallel substitutions in *Metadina trichotoma* and the three sampled ITS putative pseudogenes of *N. longipedunculata* and losses of some of the synapomorphies of *Neonauclea* s.s. in the ITS

putative pseudogenes of *N. longipedunculata*. In addition, our analyses support the hypothesis of multiple origins of the Bornean myrmecophytic *Neonauclea*, which have independently been exploited by at least three endemic *Cladomyrma* ant species. We postulate that both ecological and geographical opportunities have partly been responsible for the diversification of *Neonauclea* species in Southeast Asia. A thorough sampling of both the non- and myrmecophytic *Neonauclea* is required to re-evaluate all conclusions drawn on the evolution of ant–*Neonauclea* systems. Furthermore, we interpret the low level of variation in both the ETS and ITS sequence data as an indication of a recent and rapid radiation for *Neonauclea* s.s. and a recent and slow radiation for *Myrmeconauclea*. We argue that the diversification of *Neonauclea* s.s. in Southeast Asia are additionally associated with the nature of its fruits and seeds and its ability to colonize a wide range of habitats. In contrast, we postulate that the acquisition of the new morphological traits (pseudo-multiple fruits and long-tailed seeds) has allowed *Myrmeconauclea* to specialize on rheophytic habitats and its narrow ecological tolerance may have hindered its speciation.

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