EVOLUTION, POLLINATION BIOLOGY, AND BIOGEOGRAPHY OF THE GRAPE RELATIVE *LEEA* (LEEACEAE, VITALES)

by

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ABSTRACT OF THE DISSERTATION

Evolution, pollination biology, and biogeography of the grape relative *Leea* (Leeaceae, Vitales)

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Leea D. Royen ex L. is the sole member of the tropical family Leeaceae, which is closely related to the economically important grape family, Vitaceae. Both comprise the order Vitales. In spite of its affinity with the grape family, *Leea's* molecular systematics has remained unexplored. This study presents the first phylogeny (chapter 1) of Leeaceae using molecular markers to provide an evolutionary framework to understand its taxonomy, morphological evolution (chapter 2), ecology (chapter 3), and biogeography (chapter 4). Ridsdale (1974, 1976) estimated that there are 34 *Leea* species inhabiting the tropics of Africa and Asia. DNA sequences for the internal transcribed spacer (ITS) and the 5S non-transcribed spacer (NTS) were extracted and amplified from leaf material of 22 species, representing the morphological and geographical diversity of *Leea.* The ITS secondary structure for the type species, *L. aequata* L., facilitated homology assessments in ITS sequence alignments, while 5S-NTS data helped resolve terminal relationships. The concatenated matrix was used to estimate the phylogeny and divergence times and generate the topology for ancestral area reconstructions. Area optimization was also

performed on the Vitales topology estimated from previously published sequences to locate the geographic origin of Leeaceae, but either an out-of-Asia (i.e. Indochina) or out-of-India origin was inferred possible. The molecular phylogeny recovered four major clades, with the Indian/Indochinese *L. asiatica* (L.) Ridsdale (clade I) diverging 65.5 mya from the rest of the family. Its primitive trait of free stamens supports its position as the earliest-diverging clade. Clades II, III, and IV form a monophyletic group that had evolved in the Eocene (50.8 mya) in Indochina and/or West Malesia and exhibit the derived feature of fused stamens. Clade II, the spine-bearing species, is sister to Clade III, whose species have large flowers. Clade IV, which is unique in having multi-pinnate leaves and small stipules, evolved by the end of the Oligocene (25.6 mya) and comprise the polyphyletic 'species' (sensu Ridsdale) *L. guineensis* G. Don and *L. indica* (Burm. f.) Merr. nested among other morphologically discernible species. The radiation of *Leea* species mostly occurred in the Neogene (1.8-23.0 mya) during a time of dynamic geological and environmental changes in Southeast Asia. Africa and Australia were also colonized by Neogene dispersals of Asian *Leea.* Current species circumscriptions of *L. guineensis* and *L. indica* underestimate the genetic diversity of the genus and need to be revised. An updated checklist of 47 species reflecting clades recovered by the molecular phylogeny is presented including resurrected and putative new species. Field studies of three sympatric Philippine *Leea* morphospecies have revealed that habit and ecology must be considered in species circumscriptions.

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INTRODUCTION

The tropical plant genus *Leea,* named after the 18th century English nurseryman, James Lee, is the closest relative to the botanical family of the grapes, Vitaceae. It was originally described by van Royen, but was formally published by Linnaeus in 1767 with *L. aequata* designated as the type species. *Leea* was formerly placed in Sapotaceae and was thought to be related to either Meliaceae or Sterculiaceae (Nair 1968). *Leea* was also more recently associated with the Rhamnales (Cronquist 1981), until this was refuted by molecular evidence (Chase et al. 1993; Ingrouille et al. 2002).

The Angiosperm Phylogeny Group (APG 1998, 2003; Stevens 2001 onwards) considers *Leea* as a member of Vitaceae, under the subfamily Leeioideae Burmeister, with the rest of the 14 genera in subfamily Viticoideae Eaton, due to shared features such as raphides, pearl glands, phloem plastids, common corolla-stamen primordia, as well as similar wood and testa anatomy (Wen 2007a,b). However, unlike members of Vitaceae s. str., *Leea* species do not form tendrils and include erect herbs, shrubs and trees with terminal inflorescence and characteristically large stipules that protect the developing leaves. *Leea* flowers also possess ovaries with secondary septa and a distinct elaborate floral tube capped by stamens fused at the center (Ridsdale 1976; Wen 2007a,b). The stamens then detach as a coherent unit sometime during anthesis to reveal the receptive stigma (Gerrath et al. 1990; Molina, in press). Pollen is also unique compared to Vitaceae (Ridsdale 1976). Umadevi and Daniel (1991) noted the presence of trihidroxy compounds in *Leea*, a phytochemical trait lacking in the grapes.

Ridsdale (1974, 1976) cited the absence of a floral disc in *Leea,* and referred to the floral tube as staminodial. However, in their study of floral development in *L. guineensis* G. Don*,* Gerrath et al. (1990) disagreed with this interpretation of the floral tube. They asserted that this is a true floral disc, homologous to that of the Vitaceae, with the same structural origin, only "much more elaborate" in *Leea* (Gerrath et al. 1990: 219) and this includes the development of fused anthers, which are free in Vitaceae s. str.

These morphological differences have been acknowledged as sufficient to warrant segregation into its own monotypic family Leeaceae, originally described by Dumortier in 1829 (Planchon 1887; Ridsdale 1974, 1976; Latiff 2001; Wen 2007a). This treatment is adopted in this study. Leeaceae and Vitaceae comprise the order Vitales, which form one of the earliest diverging clades in the angiosperm tree of life, estimated to have originated sometime in the mid-Cretaceous (108-117 mya, Wikström et al. 2001; Anderson et al. 2005).

Leea species are distributed throughout the old world tropics of Africa, Madagascar, India, Indochina (i.e. Vietnam, Cambodia, Laos, Thailand, and Myanmar), Malesia (i.e. Malaysia, Indonesia, Brunei, Singapore, Philippines, and Papua New Guinea), Australia, Micronesia, and Melanesia (Ridsdale 1976; Wen, 2007a). To complicate *Leea's* ambivalent family status, there are also various opinions regarding *Leea's* species number, from 34 (Ridsdale 1974, 1976) to 70 (Ingrouille et al. 2002), to as many as 153 (Li 1998). Two species encompass vast geographical distributions, with the red-flowered *Leea guineensis* extending from tropical Africa to as far east as Palau, while the whitish/greenish-flowered *Leea indica* (Burm. f.) Merr. spanning India and Southeast Asia and the Pacific islands of Fiji and Solomon, and possibly Tonga (Ridsdale 1976).

Both species are morphologically variable, from glabrous small shrubs to pubescent small trees, and this has previously led to the creation of several segregate species. However, intermediate entities have prompted Ridsdale (1976) to combine many morphospecies into species complexes. Though most *Leea* species display compound leaves, the degree of leaf pinnation is extremely variable and dependent on age (Gerrath and Lacroix 1997), which makes it taxonomically unreliable. The subjectivity inherent in taxonomic studies of herbarium specimens, compounded by their frequent lack of pertinent morphological (color, habit) and ecological (pollination and dispersal biology) information, may result in inaccurate species circumscriptions. Thus, a molecular phylogeny becomes an invaluable tool in disentangling species complexes, which may underestimate genetic diversity because of undetected cryptic species (Bickford et al. 2007). Morphological similarity of cryptic species may be a consequence of strong stabilizing selection on optimal phenotypes (Sotuyo et al. 2007).

Taxonomic uncertainty presents as a problem not only in biodiversity estimates, but also in harnessing *Leea's* potential phytotherapeutic properties. *Leea* has been used by Asians and Africans in traditional medicine for its cardiac, analgesic and anti-inflammatory activity (Johnson 1999; Op de Beck et al. 2000, 2003). Sulphated flavonoids have been isolated in leaves of *L. guineensis,* and these have been found to act as antioxidants and inhibitors of aldose reductase, which has been implicated in the etiology of diabetes and stroke (Op de Beck et al. 1998; Thomas et al. 2000). Gupta and Chopra (1953) demonstrated *Leea's* tuberculostatic activity. The leaf extracts of *L. coccinea* Bojer (synonymized by Ridsdale with *L. guineensis*) was also effective in suppressing the growth of *E. coli* and was lethal to *Bodo*, a protozoan related to

Trypanosoma, the causative agent of the African sleeping sickness (Joseph and Molina, unpubl. data). It is critical to determine which taxonomic species actually possess pharmaceutical potential as species complexes may confound scientific testing.

This study presents the first phylogenetic investigation of Leeaceae (chapter 1) using sequence data from the internal transcribed spacer (ITS) and 5S non-transcribed (NTS) spacer, which have been extensively used in elucidating infra-familial relationships in plant taxa (Baldwin et al. 1995). To improve homology assessments, the ITS data were aligned according to the secondary structure model of *L. aequata* which was inferred using a program that optimizes folding of the primary sequence based on thermodynamic criteria (Mathews and Turner 2006). Because of their functional importance secondary structures are conserved throughout life, maintained by compensatory mutations (Hillis and Dixon 1991), and may serve as anchor points particularly in aligning ambiguous regions (Kjer 1995). Sequence data from the faster evolving 5S-NTS augmented ITS data and helped resolve cryptic species complexes, which may result from rapid radiation (Sotuyo et al. 2007).

The molecular phylogeny provided the scaffold to understand *Leea's* morphological evolution, ecology, and biogeography. In chapter 2, morphological traits such as stipule size, flower and fruit dimensions, degree of pubescence and pinnation, and flower color were examined and mapped on the topology, as well as correlated with habitat types (i.e. grasslands or seasonally dry forests vs. rainforests; islands vs. continental settings) to understand evolutionary trends in morphology and their ecological significance. In chapter 3, three sympatric *Leea* morphospecies were investigated in a natural forest plot in the Philippines to assess how floral ecology may

influence speciation. Finally, in chapter 4, the molecular phylogeny is calibrated with fossil dates to ascertain divergence times, and these were interpreted within the context of putative ancestral areas that were optimized on the topology using a parsimony-based approach.

CHAPTER 1: Phylogeny of the grape relative *Leea* (Leeaceae) inferred from nuclear ribosomal sequences

ABSTRACT

The monotypic paleotropical Leeaceae is sister to the economically important grape family, Vitaceae, but its molecular systematics and evolution are poorly known. The phylogeny of the family was elucidated using 5S-NTS and the secondary of the internal transcribed spacer (ITS) to provide a framework in which to assess current species circumscriptions and to understand *Leea's* biogeography. ITS1 and ITS2 secondary structure models for the type species, *Leea aequata,* facilitated homology assessments during alignment and were comparable to those of the Vitaceae outgroups and other phylogenetically distant taxa. In both parsimony and Bayesian analyses, four major clades were recovered with the Indian/Indochinese species of *L. asiatica* (clade I), exhibiting the plesiomorphic feature of free stamens, sister to the rest of the family (clade II-IV). Clade II is comprised of members bearing spines on the trunk and/or branches and is shown in the parsimony analysis as sister to clade III, whose Malesian members exhibit relatively larger floral and vegetative dimensions. In the Bayesian analysis, clades II and III were collapsed into a trichotomy with clade IV, which was resolved into four subclades (a-d) each of which including morphologically cryptic entities of *L. indica* and/or *L. guineensis* sensu Ridsdale (1976), along with six other morphologically discernible species. The polyphyly of *L. indica* and *L. guineensis* call for a revision of current species circumscriptions to reflect the genetic diversity of *Leea.*

INTRODUCTION

Leea D. Royen ex L. is the closest relative to the grape family, Vitaceae, which includes one of the most economically important fruit crops in the world. However, unlike the grape family, *Leea's* systematics and evolution are poorly known, which is unfortunate since this tropical plant has been used ethnobotanically for its cardiac, analgesic, tuberculostatic properties (Op de Beck 2003). *Leea*'s taxonomic status as a family is also controversial. It has been included within Vitaceae (APG 1998, 2003; Ingrouille et al. 2002) or placed into its own monotypic family, Leeaceae Dumort. (Planchon 1887; Ridsdale 1974, 1976; Latiff 2001, Wen 2007a,b). Regardless of its familial placement, *Leea* and Vitaceae s. str. (excluding *Leea*), comprise the order Vitales, which until now, has an ambivalent position in the tree of life, switching alliances with the Caryophyllales (Chase et al. 1993), Saxifragaceae (Savolainen et al. 2000), Dilleniaceae (Hilu et al. 2003) or the Rosids (Soltis et al. 2003; Jansen et al. 2006).

In spite of *Leea's* close relationship with the grape family, there has been no phylogeny developed for this tropical plant group. The aim of this study is to elucidate the evolutionary relationships within *Leea* by reconstructing its phylogeny using molecular markers. The phylogenetic framework allows the evaluation of taxon delimitations and helps develop biogeographic hypotheses on the diversification of *Leea* across the paleotropics.

The internal transcribed spacer (ITS) and the faster-evolving 5S non-transcribed spacer (5S-NTS), derived from the nuclear ribosomal DNA (nrDNA), are used to resolve

the phylogeny of *Leea.* The ITS region is composed of two spacers, ITS1 and ITS2 flanking the shorter 5.8S region. Both ITS spacers are sandwiched between the 18S and 26S ribosomal subunits (Baldwin et al. 1995). ITS and 5S-NTS are arranged in tandemly repeated units of several copies that occur in one or more chromosomal sites. Multiple copies of rDNA arrays within individuals and within species undergo concerted evolution reducing intra-individual and intra-specific variation in both homologous and non-homologous chromosomes (Hillis and Dixon 1991).

Homology assessments prior to phylogenetic analysis may be improved by aligning sequences following secondary structures that have been conserved across taxa due to their functional importance (Hillis and Dixon 1991; Kjer 1995). Consequently, *Leea* ITS is aligned following secondary structural information inferred from the method of free energy minimization (FEM), which assumes that the most optimal RNA conformation has the lowest folding free energy (Mathews and Turner 2006). The generated ITS1 and ITS2 secondary structural models of the type species, *Leea aequata* L., are also presented here, which may be used as reference to guide ITS alignments of related taxa.

MATERIALS AND METHODS

Taxon Sampling

Ninety accessions from 22 *Leea* species, representing the morphological and geographic diversity of the genus, were sampled (Table 1). *Leea* vouchers were identified following Ridsdale's keys (1974, 1976). Six additional accessions from Vitaceae (4

species; sequences downloaded from Genbank), Dilleniaceae (1 species), and Saxifragaceae (1 species, from Genbank) were included as outgroups (Table 1). Leaf material was either obtained from herbarium specimens when permission was granted by the lending institution or from silica-dried material collected by myself or donated by colleagues (S. Yap, J. Wen) and botanic gardens (University of Connecticut, University of Copenhagen, and Singapore Botanic Garden). I have tried to include sequences from *L. alata* Edgew., *L. grandifolia* Kurz, *L. simplicifolia* Zoll. & Mor., *L. tetramera* Burtt, and *L. thorelii* Gagnep., but repeated DNA extraction efforts on herbarium specimens were futile. Destructive sampling on specimens of *L. gonioptera* Laut., *L. tinctoria* Baker, and *L. unifoliata* Merr. were prohibited and thus, were not sampled. *Leea curtisii* King*, L. krukoffiana* Ridsdale*, L. saxatilis* Ridl., and *L. smithii* Koord. were not sampled due to unavailability of material.

DNA extraction, amplification and sequencing

DNA was extracted following a modified CTAB protocol used for Vitaceae (Soejima and Wen 2006). Leaves were first pulverized with sand at room temperature and incubated in 4x CTAB buffer mixed with 2% mercaptoethanol, 2% polyvinylpyrrolidone (PVP), 2% polyethylene glycol (PEG), and 1% sodium bisulfite at 60^{o}C for 2 hrs. DNA was purified with chloroform:isoamyl alcohol (24:1) twice, precipitated with isopropanol and 5M NaCl at -20^{o}C overnight then washed with 95% ethanol. NaOAc (3M, pH=4.8) was added to the pellet for final precipitation then washed twice with 70% ethanol. Samples were air-dried and maintained in TE buffer at -20^{o}C for short term storage. Since the entire ITS region, which was >700 bp long, cannot be

successfully amplified, primer pairs for each of its shorter spacers (<300 bp) were designed: P79 (forward: AAGGATCATTGTCGARCCYGCA)-P80 (reverse: AGATATCCGTTGCCGAGAGTC) for ITS1 and P81 (forward: ACGACTCTCGGCA-ACGGATATCT)-P82 (reverse: ATGCTTAAACTCAGCGGGTGTTCC) for ITS2. 5S-NTS was amplified using a nested PCR approach, initially with the forward primer CACCGGATCCCATCAGAACT and the reverse primer TTAGTGCTGGTATGAT-CGCA (Udovicic et al. 1995) and then with the internal primers TTGGGAAGTYYCY TGTGTTGCA (forward) and TGGTATGATCGCACCCRTCATG (reverse) designed specifically for *Leea*.

Amplification reactions were performed in a 25 uL volume containing Choice Taq Mastermix™ (1.5 mM $MgCl_2$, 10 mM Tris-HCl at pH 9.0, 10 mM KCl, 8 mM $(NH_4)_2SO4$, 0.05% Triton X-100, dNTP mix; Denville Cat. No. CB4070-7), 0.7 uM primer, 0.05 ug/uL BSA, 5% DMSO, 0.8 M Betaine, additional $MgCl_2$ (until 2.5 mM final concentration) and 3 uL template, which was prepared by diluting the CTAB extracts 1:50. PCR reactions were conducted on Applied Biosystems GeneAmp System 9700. PCR amplification profile for both ITS regions was as follows: 97^{o}C for 1 min, followed by 35 cycles of 95^{o}C for 1 min, 53^{o}C for 1 min, and 68^{o}C for 2 min, ending with a final extension of 72 ^{o}C for 4 min. The PCR program for 5S-NTS started at 94^{o}C for 2 min, followed by 27 cycles of 94^{o}C for 1 min, 60^{o}C for 1 min, and 72^{o}C for 1 min, ending with a final extension of 72 ^{o}C for 4 min.

For visualization, PCR products were run on 1% agarose gel. If double bands were observed, the desired fragment was cut out of the gel and treated with QIAEX II Gel Extraction Kit (Qiagen cat. #20051) to yield cleaned DNA that may be used for a

second PCR reaction. PCR products that resulted in single bands were cleaned using ExoSAP-IT® (USB cat.# 78201) following manufacturer's specifications, and then submitted to Genewiz Inc. for sequencing. Each DNA fragment (ITS1, ITS2, 5S-NTS) was sequenced in both directions.

Alignment and Secondary Structure Prediction

Both ITS and 5S-NTS sequences were assembled and trimmed in Sequencher ver. 4.6 (Gene Codes Corp.) and aligned with ClustalW (European Bioinformatics Inst.) using default parameters. Gaps were coded with Gapcoder (Young and Healy 2003). ITS1 and ITS2 sequences were then manually adjusted in Microsoft Word following secondary structure information generated by the software RNAstructure v. 4.5 (Mathews et al. 2004) using the function "Fold RNA single strand". The output consisted of .ct files, which stores the secondary structure information for each sequence. Twenty structures are stored in each .ct file, with the first one presented having the lowest free energy, and may be the most probable structure, while the other 19 are alternate hypotheses sampled heuristically (Mathews, pers. comm.). Base-pairing was saved as a text file (also known as a helix file).

Base-pairing probabilities for helices (alternatively called stems) in the secondary structure predictions were calculated using RNAstructure's partition function tool (Mathews and Turner 2006), which also allows color-annotation of putative stems. Detailed methods are available in Molina and Struwe (in press). On average, 91.0% of base pairs with a probability of 0.99 or greater of pairing ($P_{BP} \geq 0.99$) are correctly predicted based on comparative sequence analysis (Mathews 2004), while only 83% of

base pairs with $P_{BP} \geq 0.90$ may be correctly predicted. The helix files previously generated were modified to retain only highly probable base pairs ($P_{BP} \geq 0.90$). These were exported into XRNA (B. Weiser and H. Noller, University of California, Santa Cruz) to draw secondary structure models for ITS1 and ITS2.

The ITS (ITS1, ITS2) and 5S-NTS sequence data were concatenated to produce an alignment consisting of 920 bp, which includes coded gaps that were treated as standard characters in both parsimony and Bayesian analyses.

Phylogenetic Analyses

The alignment was converted into a nexus file. Phylogenetic inference was conducted in PAUP* v4.0 (Swofford 2003) and MrBayes V3.1.2 (Huelsenbeck and Ronquist 2001). An equally-weighted parsimony analysis was conducted in PAUP invoking 500 nonparametric bootstrapping (BS) replicates, each with 20 random addition-sequence replicates (rearrlimit=1,000,000 limitperrep=yes, MULTREES on). To generate the parameters implemented in the Bayesian analyses, MrModeltest 2.2 (Nylander 2004) was used, which called for a GTR model with uniform state frequencies and rates set to gamma. Two independent runs of one million iterations each were performed (nchains=4) resampling trees every 500 generations. The log likelihoods were plotted to identify the point where log likelihood has stabilized, and trees prior to this point were discarded as burnin. The trees were then pooled and imported into PAUP ver. 4.0b10 (Swofford 2003), and the majority rule consensus tree was obtained to determine the posterior probabilities of the clades.

RESULTS

Alignment and secondary structure

Multiple peaks corresponding to intragenomic polymorphisms were seldom detected in the sequencing profile, suggesting that ITS sequences obtained have been sufficiently homogenized. *Leea* ITS1 and ITS2 ranged from 279-301 bp, 253-262 respectively. The GC content was 67.8%**.** A few multiple peaks were encountered in some 5S-NTS sequences. These nucleotides were assigned ambiguity codes. 5S-NTS ranged from 186-374 bp, with a GC content of 65.0%.

The ITS alignment was refined based on the secondary structural models predicted by free energy minimization in RNAstructure v4.5. These models are illustrated for ITS1 and ITS2 of the type species, *Leea aequata* (Fig. 1). Base pairs with pairing probabilities greater than or equal to 90% and/or supported by the presence of compensatory mutations are depicted.

Phylogeny

The most parsimonious trees from the concatenated dataset are 1157 steps in length, with a consistency index (CI) of 0.49, and a retention index (RI) of 0.72. The strict consensus tree is presented in Fig. 2. Constant sites totaled 482, while 610 characters were considered parsimony-informative. *Leea* is monophyletic with four major clades (I-IV), which was also reflected in the Bayesian phylogeny (Fig. 3). However, the topologies dispute in the placement of the major clades, as well as in the positioning of the subclades within clade IV.

Clade I is represented by *L. asiatica* sensu Ridsdale (1974), which is well-supported in both analyses. Clade II, which is also robustly supported, includes spine-bearing *Leea* species. This clade is contentious in its position, being recovered as sister to Clade III in the parsimony analysis with strong support (at least 85% BS), but shown as part of a trichotomy with clades II and IV in the Bayesian estimation. Clade III is comprised of species that possess relatively large floral and vegetative dimensions. The species complexes *L. indica* and *L. guineensis* are neither monophyletic and fall in Clade IV in both analyses, along with other distinct morphospecies--*L. aequata, L. compactiflora, L. macrophylla, L. rubra,* and *L. setuligera.*

In the Bayesian topology (Fig. 3), four strongly supported (>90% p.p.) subclades (a-d) occur in Clade IV , but subclade "a" was not recovered in the parsimony analysis (Fig. 2). *Leea indica* from New Guinea, Northern Australia, and the Pacific Islands of Fiji and Solomon cluster in subclade "a" in the Bayesian topology (Fig. 3). This subclade is sister to predominantly Indochinese (i.e. Vietnam, Cambodia, Laos, Thailand, and Myanmar) and Malesian (i.e. Malaysia, Indonesia, Brunei, Singapore, Philippines, and Papua New Guinea) *Leea* species (subclades b-d, Figs. 3, 4). Subclade "b", corresponding to *L. macrophylla,* occurs in India, Indochina, and tropical China. Subclades "c" and "d" are sisters, with subclade "c" represented by species from India and Indochina (*L. setuligera, Leea* sp. *KR37301, Leea* sp. *KR975877*; Table 1). Embedded within subclade "c" are also *L. guineensis* s. l. from Mauritius, Madagascar, and tropical Africa, which form a monophyletic group. Subclade "d" includes predominantly Malesian species that extend to India and Indochina (*L. aequata*, *L. indica* s.l., *L. rubra*), tropical Australia (*L. rubra*), and Caroline Islands (*L. guineensis* s.l.).

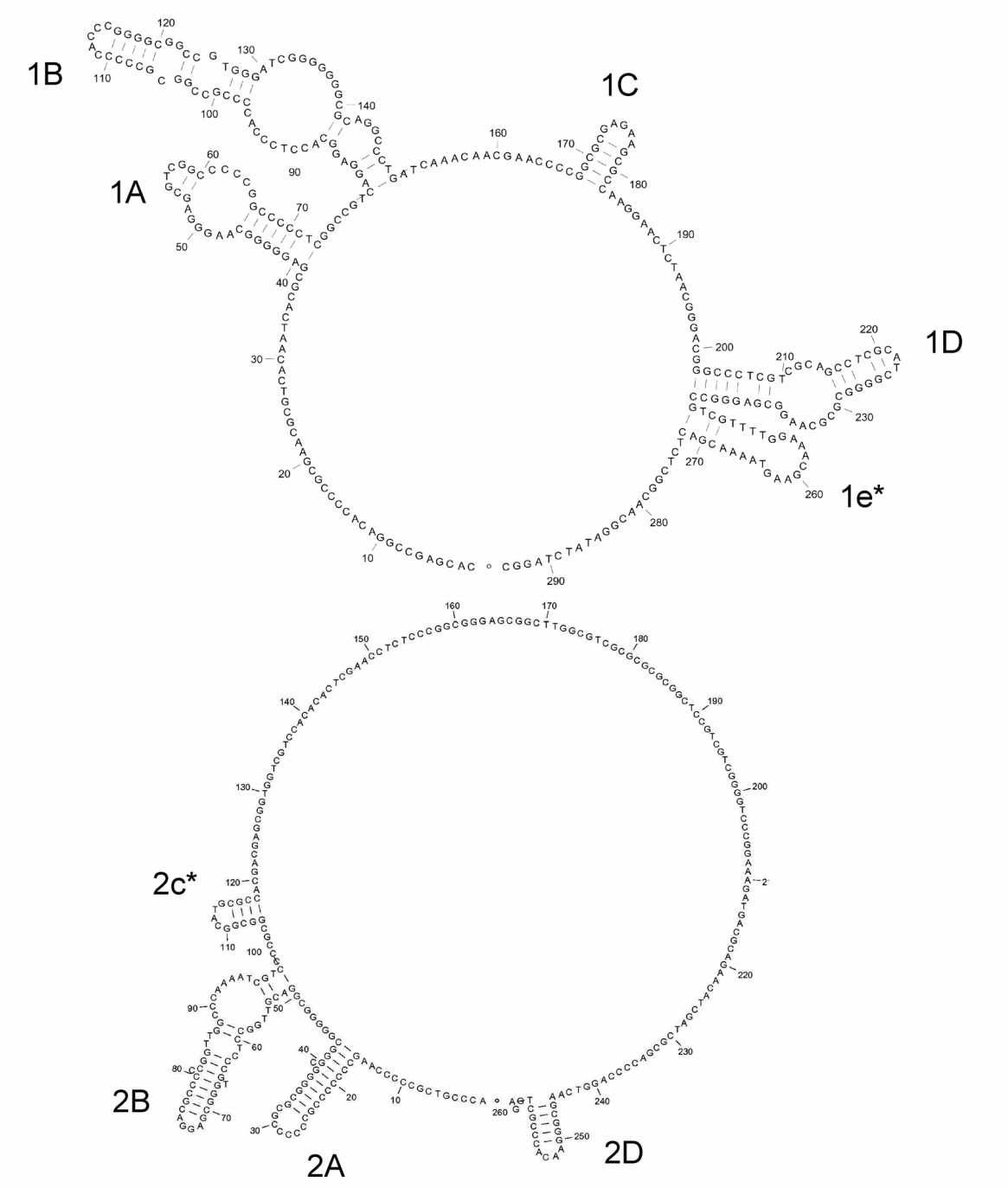

Fig. 1. Structural models of ITS in *Leea aequata*. Motifs homologous to stems in other eudicots are labeled following Goertzen et al.'s (2003) annotations. A. Model of ITS1. Stems 1A-1D are homologous to stems in other eudicots examined, except for stem 1e*, which was only present in *Tetrastigma* (Vitaceae). B. Model of ITS2. Stems 2A, 2B, and 2D are homologous to stems in other eudicots. 2c* represents a stem not homologous to Goertzen et al.'s 2C.

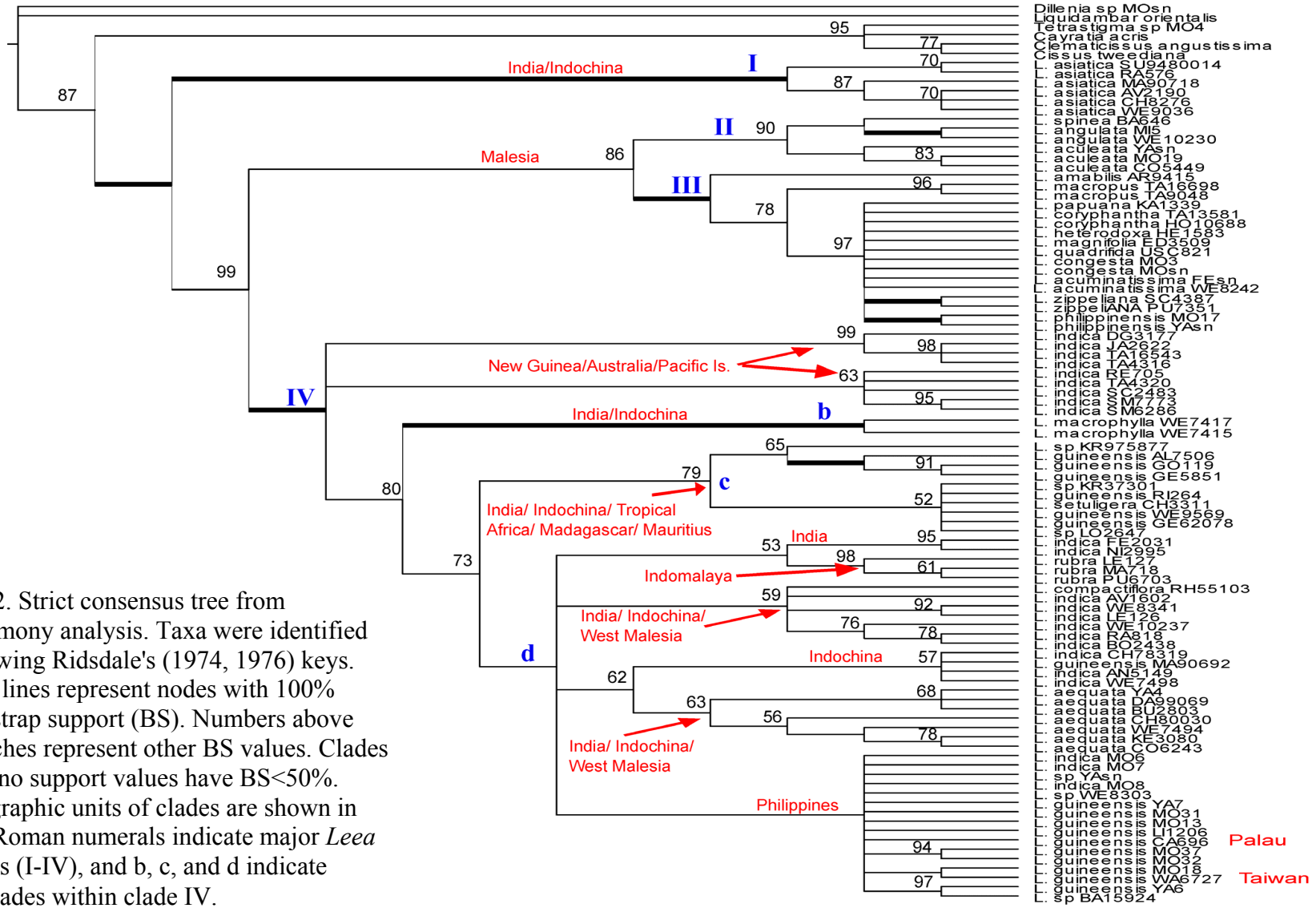

Fig. 2. Strict consensus tree from parsimony analysis. Taxa were identified following Ridsdale's (1974, 1976) keys. Bold lines represent nodes with 100% bootstrap support (BS). Numbers above branches represent other BS values. Clades with no support values have BS<50%. Geographic units of clades are shown in red. Roman numerals indicate major *Leea* clades (I-IV), and b, c, and d indicate subclades within clade IV.

DISCUSSION

Secondary structure

Past studies have affirmed the phylogenetic utility of the ITS region (Baldwin et al. 1995), but only a few studies have employed its secondary structure as the guide for alignment in plant taxa, which may considerably improve phylogenetic estimation (Goertzen et al. 2003 for Asteraceae; Bellarosa et al. 2005 for *Quercus,* Fagaceae; Campbell et al. 2005 for *Picea,* Pinaceae; Molina and Struwe, in press, for tribe Potalieae, Gentianaceae; Frasier and Struwe, in press for *Strychnos,* Loganiaceae).

Free energy minimization (FEM) identified five helices (1A, 1B, 1C, 1D, 1e*; Fig. 1A) in ITS1 of *L. aequata* that are well-supported (i.e. bp probability at least 90%) by the partition function calculation of RNAstructure v4.5. The first four correspond to stems found in ITS1 of the outgroups *Dillenia* and *Liquidambar* used in this study (not shown, but available from author upon request). These stems were also identified in Gentianaceae (Molina and Struwe, in press), *Strychnos* (Frasier and Struwe, in press) and in *Anvillea radiata* (Asteraceae), though 1D was lacking in the structural model of *Anvillea* (Goertzen et al. 2003). *Leea aequata* stems 1A-1D also correspond to the last four helices (II-VI) of *Quercus* (Fagaceae, Bellarosa et al. 2005). Nucleotides in stems 1A, 1B, 1D between the outgroups and the *Leea* ingroup could not be aligned unless the stems were marked in the primary sequence, thus the stems provided anchor points by which sequences may be effectively aligned in spite of nucleotide differences. Stem 1C was readily alignable as this pertains to the Universal Core Motif in angiosperms (Liu and Schardl 1994).

Leea stem 1e* was not identified in the asterid models mentioned above, nor in the outgroups *Dillenia* or *Liquidambar* (other core eudicots). However, 1e* is depicted in *Tetrastigma* (structure not shown), one of the Vitaceae outgroups. Stem 1e* may also be present in the other Vitaceae outgroups (*Cayratia, Cissus,* and *Clematicissus*), but their sequences, obtained from Genbank (Table 1), lack the last few bases necessary to determine the occurrence of stem 1e*. To determine if stem 1e* is present in other rosid taxa, the ITS1 sequences of *Aronia* sp. (EF127043, Rosaceae), *Elaeocarpus williamsianus* (DQ448691, Elaeocarpaceae) *Quercus petraea* (EU628558, Fagaceae) were obtained from Genbank (as complete ITS sequences) and were also folded using FEM in RNAstructure. Stem 1e* is so far non-existent in these randomly obtained rosid taxa (secondary structure models available from author upon request). Considering the possibility that stem 1e* occurs in Vitaceae and its presence in *Leea* suggest that this may be a conserved motif unique to the Vitales. Further comparative sequence analyses are necessary to confirm this.

In the structural model of ITS2 for *L. aequata*, four stems (Fig. 1B: 2A, 2B, 2c*, 2D) were determined to be well-supported (bp probability ≥90%). These were also identified in other eudicot taxa examined and may be homologous, except for 2c*, which did not correspond with the position of stem 2C in the other taxa including *Tetrastigma* (Vitaceae). However, when the ITS2 sequence of *L. asiatica* (*SU9480014*) was folded using the same methodology, a stem allegedly corresponding to the 2C of the other taxa was identified. Nonetheless, stem 2C was a hypervariable region, that was difficult to align among the taxa examined, in spite of the comparatively similar positioning of this

motif. Hence, this particular stem may not be under the same selective pressure as the other more conserved motifs such as stems 1C, 1D, 2B, and 2D.

The structure of 2B is almost invariant across taxa, which is a helix interrupted by a universally conserved pyrimidine bulge (Mai and Coleman 1997). In *L. aequata* (Fig. 1B), this bulge is made of unpaired TT-CT on opposite strands. The same nucleotides make up the bulge in *Tetrastigma* and *Liquidambar*, but are CC-CT in *Dillenia.* Stem 2D is relatively variable in nucleotide sequence compared to 2B, but was also easy to align after compensatory mutations were identified. This was the same pattern seen in the phylogenetically distant Gentianaceae (Molina and Struwe, in press).

The ubiquitous presence of these stems in unrelated plant groups suggest that these have been evolutionarily constrained. Considering these conserved motifs in alignments of rDNA markers has facilitated homology assessments for the following phylogenetic analyses.

Phylogeny

Four major clades (I-IV; Figs. 2, 3) were recovered in both phylogenetic analyses, but the parsimony analysis (Fig. 2) supports a sister relationship between Clades II and III with strong support (>85%). The Bayesian estimation (Fig. 3), on the other hand, collapses this relationship into a polytomy shared with clade IV.

Clade I. Clade I includes *Leea asiatica* s.l. (incl. *L. aspera,* sensu Ridsdale), which is a widespread species found in India, Bhutan, Nepal, Bangladesh, Indochina, and tropical China. This clade is robustly supported in both analyses. 5S-NTS sequences of accessions sampled for this clade share a >100-bp deletion. Morphological

synapomorphies include greenish flowers, narrow wing-like stipule traversing the length of the petiole, and moderately large fruits (c.12 mm wide) (Ridsdale 1974). In contrast to the rest of the genus, which develop a floral tube topped with anthers fused at the center, *L. asiatica*'s s.l. anthers are free. This seems to be a plesiomorphic feature since the Vitaceae also do not develop fused anthers.

Within *L. asiatica,* two clades are apparent that morphologically correspond to *L. crispa* van Royen ex L. and *L. aspera* Wall. ex G. Don, which were both synonymized with *L. asiatica* by Ridsdale (1980). The "crispa" clade is characterized by petioles and peduncles with crisped wings and acutely serrate leaves that are glabrous above *(AV2190, CH8276, MA90718, WE9036)*. The "aspera" clade *(RA576, SU9480014)*, on the other hand, differs in having leaves with white scattered appressed hairs between nerves and generally crenate-serrate margin (Lawson1875).

Clade II. Clade II includes *Leea* species that bear spines, which is a distinct synapomorphy. *Leea aculeata* is riddled with spines only on the trunk of the tree, while *L. angulata* have them on terminal branches as well. *L. aculeata* and *L. angulata* are widespread in Malesia. *Leea spinea,* which occurs in Madagascar and Comoros, is sister to *L. angulata,* and similarly bears spines on both trunks and branches (Ridsdale 1974, 1976). *L. aculeata* has only been recorded once in New Guinea (Fakfak), while *L. angulata* has not been collected there. But unlike *L. aculeata,* it is found in Thailand and Malaya, extending as far west to the Nicobar Islands (Ridsdale 1974, 1976).

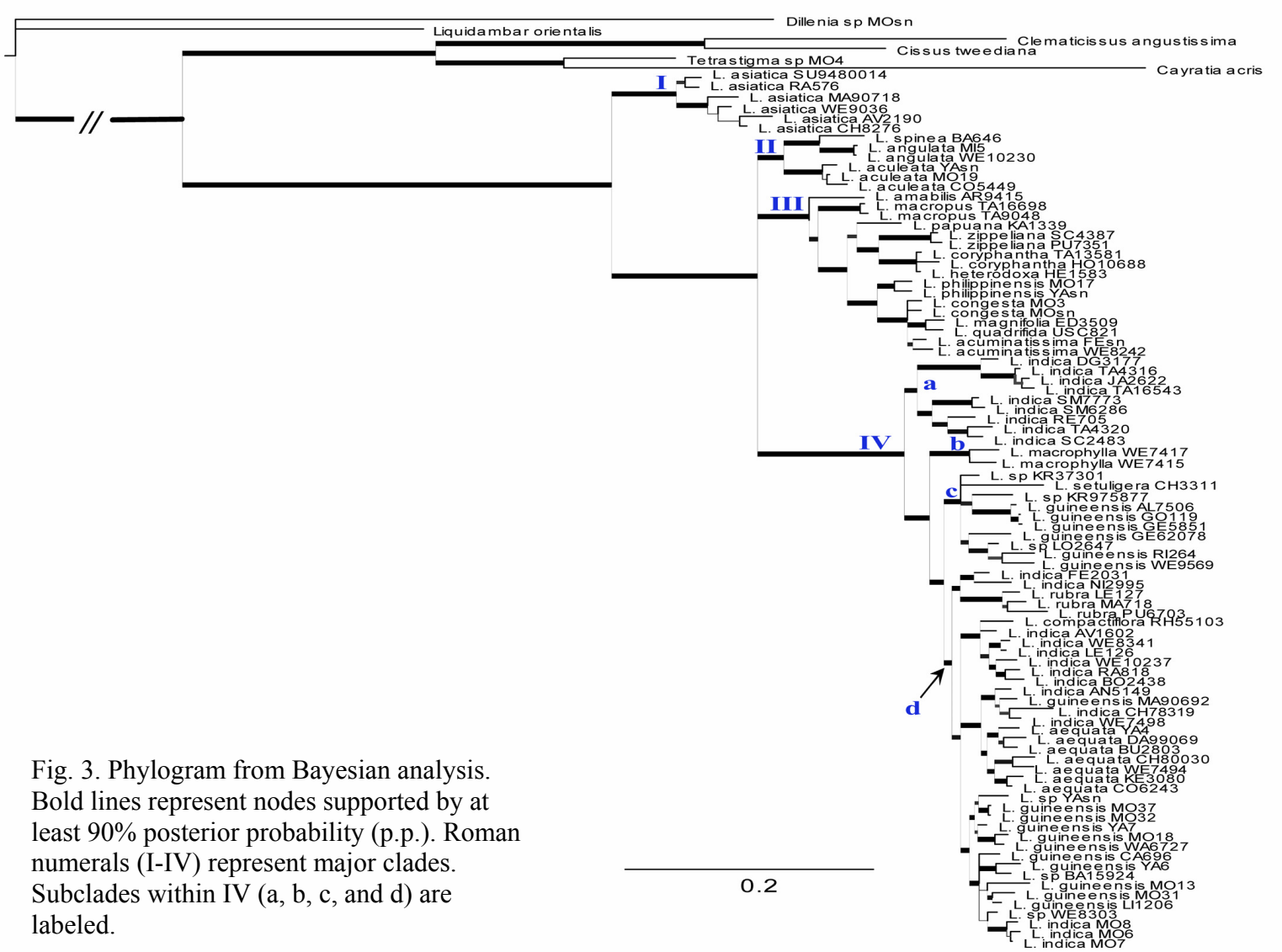

Fig. 3. Phylogram from Bayesian analysis. Bold lines represent nodes supported by at least 90% posterior probability (p.p.). Roman numerals (I-IV) represent major clades. Subclades within IV (a, b, c, and d) are labeled.

Clade III. The narrow endemic *Leea amabilis,* restricted to West Borneo, is the earliest diverging branch of Clade III, which includes *Leea* species that possess larger, thicker flowers (> 4.5 mm long in dried material), leaf dimensions (>150 mm long, >50 mm wide), and fruits (11-25 mm wide) than the other major clades. Leaves are also generally coriaceous, which is uncommon in the other *Leea* clades. *Leea macropus* from the Bismarck Archipelago is sister to a clade comprised of species from New Guinea (*L. coryphantha, L. heterodoxa, L. papuana,* and *L. zippeliana*) and the Philippines (*L. acuminatissima, L. congesta, L. magnifolia,* and *L. quadrifida*).

Though *L. tetramera* of the Solomon Islands was not included in the analysis, its morphological similarity and geographical proximity to *L. macropus* suggest its strong evolutionary affinity with the latter. The two species differ only in flower length (i.e. from corolla base to tip of floral tube), with *L. macropus'* flowers slightly longer than *L. tetramera'*s. The stipule scar is about half as long as the petiole in *L. macropus,* but almost as long as the petiole in *L. tetramera.*

The species from New Guinea within Clade III are sister to the Philippine species. The Philippine species are monophyletic, distinctive in having 4-merous flowers and 4-seeded fruits since generally elsewhere in the genus, flowers are 5-merous and fruits are 6-seeded. *Leea unifoliata,* which was not included here, most probably belongs to this clade, by virtue of its 4-merous corolla.

Due to the scarcity of material, the very rare *L. gonioptera,* recorded only in Irian Jaya, New Guinea, was not sampled. It is difficult to speculate on the evolutionary affinity of this species due to the variability of its morphs. This species is unifoliolate or pinnate, with small greenish-white flowers. The stipule extends throughout the petiole in

unifoliolate specimens, but is up to half as long as the petiole in pinnate specimens (Ridsdale 1976). Ridsdale (1976:767) attested to the difficulty of readily distinguishing the unifoliolate specimen from the strictly unifoliolate *L. zippeliana.* The small flowers (< 4.5 mm long) and fruits (9-12 mm) make its inclusion among the New Guinean species of clade III questionable.

Clade IV. Clade IV is predominantly composed of the morphologically homogeneous *L. indica/L. guineensis* complex, but also includes the morphologically distinct *L. aequata, L. compactiflora, L. macrophylla, L. rubra,* and *L. setuligera.* The presence of several distinct morphotypes in each of the smaller subclades (Figs. 3, 4; subclades a-d) call for a revision of current species circumscriptions.

Subclade "a" is sister to all other subclades and is comprised of *L. indica* entities from the Australia/New Guinea/Pacific Islands region. This clade is not supported in the parsimony analysis (Fig. 2). *Leea macrophylla* forms subclade "b" in both analyses. This entity is another complex of morphs with sympatric distributions, that may be ecologically related (Ridsdale 1974). Ridsdale recognized this as one morphospecies because they essentially possess the same floral structure, in spite of variations in leaf indument and leaf pinnation--from unifoliolate *(Wen 7415)*, i.e. *macrophylla s. str.* to pinnate *(Wen 7417)*, i.e. *robusta* Roxb. Ridsdale's opinion seems to be preliminarily supported by the association of both morphospecies in subclade "b" (Figs. 3, 4). The Malayan/Sumatran *L. simplicifolia* and the Irian Jayan *L. gonioptera* also include unifoliolate and pinnate forms, which may suggest their close affinities with *L. macrophylla,* though this needs to be confirmed phylogenetically.

The rare *Leea setuligera* from India, Thailand, and tropical China is involved in a

polytomy within subclade "c" with two undetermined species from Myanmar (*KR37301, KR975877*), and *L. guineensis* s.l. from Africa, Comoros, Madagascar, and Mauritius (Figs. 2, 3). The 'basal' (i.e. early diverging) position of Indian/Indochinese entities in subclade "c" hints on the probability of a dispersal from Asia to Africa. Though Ridsdale has included the yellow-flowered entities from Africa in *L. guineensis* s.l.*,* it is perhaps this differently colored flowers that clearly identifies this morphospecies from the rest of the complex, which has reddish-flowers. A 100-bp sequence deletion in 5S-NTS (not homologous to that of clade I) and the insertion of 3 G's in stem 2A are two unique molecular synapomorphies for this subclade. Though not included here, it is possible that the São Tomé endemic, *L. tinctoria,* may be related to the African entities, to which it is vegetatively indistinguishable. The orange flowers, however, are distinctive, being more than twice as long.

Subclade "d" is comprised of *L. aequata, L. rubra, L. compactiflora,* and the Indian, Indochinese and Malesian entities of *L. indica* and *L. guineensis*. *Leea aequata* is distinguishable from *L. indica* s.l. in possessing large pearl glands on the underside of the leaf surface. The reddish-flowered *L. rubra* is separated from *L. guineensis* by its distinctive wing-like stipules that occupy the entire petiole. *Leea guineensis* s.l. stipules, in contrast, are obovate in shape and do not span the whole petiole length. *Leea compactiflora* is similar vegetatively to both *L. indica* and *L. guineensis,* but differs in having large conspicuous bracts on its flowers.

The polyphyly of both *L. indica* and *L. guineensis* throughout Clade IV strongly indicates that these are species complexes composed of several cryptic and possibly rare endemics, and that flower color is homoplastic and unreliable for species definitions. It is

notable that many entities in this clade exhibit geographic cohesion (Fig. 2), which indicates that diversification within subclades was triggered by the complex geological events that had defined Southeast Asia during the Tertiary epoch (Molina, chapter 4). Noteworthy geographic units include: Australia/New Guinea/Pacific Islands (subclade "a"); India/Indochina (subclade "b"); Africa/Madagascar/Mauritius embedded within India/Indochina (subclade "c"); Philippines, including Taiwan and Palau (Caroline Islands), suggesting dispersal to the latter areas (within subclade "d"); West Malesia (Malay Peninsula, Borneo, Sumatra, Java, Palawan); and the more encompassing Indomalaya, which includes India, Indochina, and the entire Malesian region (West Malesia including the Philippines and East Malesia, i.e. New Guinea).

The current species circumscriptions need to be revised to reflect the inherent genetic diversity of the genus. Though molecular data show resolution among clades, the lack, or at least obscurity, of distinctive morphological synapomorphies render revisionary studies challenging. Thus, it is difficult to surmise the evolutionary affiliations of species that were not sampled *(L. alata, L. curtisii, L. grandifolia, L. krukoffiana, L. simplicifolia, L. thorelii, L. saxatilis, L. smithii)* in this analysis due to the unavailability of material. Even Ridsdale (1974, 1976) questioned the species status of *L. curtisii, L. krukoffiana, L. saxatilis*, and *L. smithii* because there were few collections to examine and their types lacked pertinent structures. It is clear, however, that a visual survey of over 900 herbarium sheets has presented more morphospecies than what are presently described (J. Molina, pers. obs.). The unreliability of morphological data to infer evolutionary kinships necessitate a more extensive phylogeographic analyses to ascertain the species status, geographic limits, and relationships of these entities.

Table 1. Accessions and sequences used in phylogenetic analyses including voucher information (collector, number, provenance, and herbarium source). Taxa were identified following Ridsdale's treatments (1974, 1976), except for the outgroups (*Cayratia acris, Cissus tweediana, Clematicissus angustissima, Dillenia* sp., *Liquidambar styraciflua, Tetrastigma*), whose Genbank accession numbers are indicated.

Taxon	**Collector, collector number**	**Provenance**	**Herba-rium**	**Sequence available**
Cayratia acris F. Muell. (Vitaceae)	--	--	--	ITS1 (AF365985)
Cissus tweediana (Baker) Planch. (Vitaceae)	--	--	--	ITS1 (AY998779)
Clematicissus angustissima (F. Muell.) Planch. (Vitaceae)	--	--	--	ITS1 (AY037913)
Dillenia sp. L. (Dilleniaceae)	Molina, s.n.	Palanan, Philippines	CHRB	ITS1
Leea aculeata Blume ex Spreng.	Coode et al. 5449	Mindoro, Philippines	L	ITS1, 5snts
Leea aculeata Blume ex Spreng.	Molina 19	Palanan, Philippines	CHRB	ITS, 5snts
Leea aculeata Blume ex Spreng.	Yap s.n.	Laguna, Philippines	CHRB	ITS1, 5snts
Leea acuminatissima Merr.	Ferrerras s.n.	Aurora, Philippines	PUH	ITS, 5snts
Leea acuminatissima Merr.	Wen 8242	Laguna, Philippines	US	ITS, 5snts
Leea aequata L.	Burley et al. 2803	West Kalimantan, Indonesia	L	ITS1, 5snts
Leea aequata L.	Chow and Wan 80030	Yunnan, China	MO	ITS1, 5snts
Leea aequata L.	Coode 6243	Sulawesi, Indonesia	A	ITS1, 5snts
Leea aequata L.	Davies 99069	Borneo	A	ITS, 5snts
Leea aequata L.	Kessler 3080	Sulawesi, Indonesia	L	ITS, 5snts
Leea aequata L.	Wen 10172	Sulawesi, Indonesia	US	5snts
Leea aequata L.	Wen 7494	Thailand	US	ITS, 5snts
Leea aequata L.	Yap 4	Bohol, Philippines	CHRB	ITS, 5snts
Leea amabilis Veitch ex Mast.	Argent and Wilkie 9415	Central Kalimantan, Indonesia	A	ITS, 5snts
Leea angulata Korth. ex Miq.	Mitchell 5	Christmas Island, Australia	CANB	ITS1, 5snts
Leea angulata Korth. ex Miq.	Wen 10230	Sulawesi, Indonesia	US	ITS, 5snts
Leea asiatica (L.) Ridsdale	Averyanov et al. 2190	Vietnam	MO	ITS1, 5snts
Leea asiatica (L.) Ridsdale	Chand 8276	Assam, India	MICH	ITS
Leea asiatica (L.) Ridsdale	Maxwell 90718	Thailand	A	ITS1
Leea asiatica (L.) Ridsdale	Wen 9036	China	US	ITS, 5snts
Leea asiatica (L.) Ridsdale	Ram 576	West Nepal	A	ITS1
Leea asiatica (L.) Ridsdale	Suzuki et al. 9480014	Central Nepal	A	ITS, 5snts
Leea compactiflora Kurz	Averyanov et al.1602	Vietnam	MO	ITS, 5snts
Leea compactiflora Kurz	Hui 55103	China	US	ITS

Table 1 (cont'd.)

Leea congesta Elmer	Molina 3	Palanan, Philippines	CHRB	ITS, 5snts
Leea congesta Elmer	Molina s.n.	Palanan, Philippines	CHRB	ITS, 5snts
Leea coryphantha Lauterb.	Hoogland and Craven 10688	Papua New Guinea	CANB	ITS2, 5snts
Leea coryphantha Lauterb.	Takeuchi et al. 13581	Papua New Guinea	A	ITS2
Leea guineensis G. Don.	Alcool 7506	Congo, Africa	NY	ITS, 5snts
Leea guineensis G. Don.	Canfield 696	Palau, Caroline Islands	US	ITS
Leea guineensis G. Don.	Gereau et al. 5851	Tanzania, Africa	MO	ITS, 5snts
Leea guineensis G. Don.	Gentry and Schatz 62078	Madagascar	MO	ITS, 5snts
Leea guineensis G. Don.	Gobbo 119	Tanzania, Africa	MO	ITS, 5snts
Leea guineensis G. Don.	Molina 13	Palanan, Philippines	CHRB	ITS, 5snts
Leea guineensis G. Don.	Molina 18	Palanan, Philippines	CHRB	ITS, 5snts
Leea guineensis G. Don.	Molina 31	Palanan, Philippines	CHRB	ITS, 5snts
Leea guineensis G. Don.	Molina 32	Negros, Philippines	CHRB	ITS, 5snts
Leea guineensis G. Don.	Molina 37	Negros, Philippines	CHRB	ITS, 5snts
Leea guineensis G. Don.	Liao 1206	Taiwan	A	ITS, 5snts
Leea guineensis G. Don.	Maxwell 90692	Thailand	A	ITS1, 5snts
Leea guineensis G. Don.	Richards and Von Bargen 264	cultivated	DBG	ITS, 5snts
Leea guineensis G. Don.	Wagner 6727	Taiwan	F	ITS1, 5snts
Leea monticola WE9569	Wen 9569	Madagascar	US	ITS, 5snts
Leea guineensis G. Don.	Yap 6	Leyte, Philippines	CHRB	ITS1, 5snts
Leea guineensis G. Don.	Yap 7	Surigao del Sur, Philippines	CHRB	ITS, 5snts
Leea heterodoxa K. Schum. & Lauterb.	Heyligers 1583	Papua New Guinea	CANB	ITS1, 5snts
Leea indica (Burm. f.) Merr.	Anderson 5149	Thailand	A	ITS, 5snts
Leea indica (Burm. f.) Merr.	Bourell 2438	Palawan, Philippines	A	ITS, 5snts
Leea indica (Burm. f.) Merr.	Fell and Stanton 3177	Queensland, Australia	CANB	ITS, 5snts
Leea indica (Burm. f.) Merr.	Fernandes 2031	Bombay, India	A	ITS1, 5snts
Leea indica (Burm. f.) Merr.	Jackes 2622	Queensland, Australia	JCT	ITS, 5snts
Leea indica (Burm. f.) Merr.	Molina 6	Palanan, Philippines	CHRB	ITS, 5snts
Leea indica (Burm. f.) Merr.	Molina 7	Palanan, Philippines	CHRB	ITS, 5snts
Leea indica (Burm. f.) Merr.	Molina 8	Palanan, Philippines	CHRB	ITS, 5snts
Leea indica (Burm. f.) Merr.	Lee 126	Singapore	SING	ITS, 5snts
Leea indica (Burm. f.) Merr.	Nicolson 2995	Mysore, India	US	ITS, 5snts
Leea indica (Burm. f.) Merr.	Ramadhanil and Schultze 818	Sulawesi, Indonesia	CANB	ITS, 5snts
Leea indica (Burm. f.) Merr.	Regalado et al. 705	Solomon Islands	CANB	ITS
Leea indica (Burm. f.) Merr.	Schodde 2483	Papua New Guinea	A	ITS1

Table 1 (cont'd.)

Leea indica (Burm. f.) Merr.	Smith 6286	Viti Levu, Fiji	US	ITS1
Leea indica (Burm. f.) Merr.	Smith 7773	Ngau, Fiji	US	ITS, 5snts
Leea indica (Burm. f.) Merr.	Takeuchi and Ama 16543	Papua New Guinea	A	ITS1, 5snts
Leea indica (Burm. f.) Merr.	Takeuchi 4316	Papua New Guinea	F	ITS, 5snts
Leea indica (Burm. f.) Merr.	Takeuchi and Wiakabu 4320	Papua New Guinea	CANB	ITS, 5snts
Leea indica (Burm. f.) Merr.	Wen 10237	Sulawesi, Indonesia	US	ITS, 5snts
Leea indica (Burm. f.) Merr.	Wen 7498	Thailand	US	ITS, 5snts
Leea indica (Burm. f.) Merr.	Wen 8341	Malaysia	US	ITS, 5snts
Leea indica (Burm. f.) Merr.	Chow 78319	Hainan, China	A	ITS
Leea macrophylla Roxb. ex Hornem.	Wen 7415	Thailand	US	ITS, 5snts
Leea macrophylla Roxb. ex Hornem.	Wen 7417	Thailand	US	ITS, 5snts
Leea macropus K. Schum. & Lauterb.	Takeuchi 16698	Papua New Guinea	A	ITS
Leea macropus K. Schum. & Lauterb.	Takeuchi 9048	Papua New Guinea	A	ITS1, 5snts
Leea magnifolia Merr.	Edano 3509	Mindoro, Philippines	MICH	ITS1
Leea papuana Merr. & L. M. Perry	Kanis 1339	Papua New Guinea	CANB	ITS, 5snts
Leea philippinensis Merr.	Molina 17	Palanan, Philippines	CHRB	ITS, 5snts
Leea philippinensis Merr.	Yap s.n.	Samar, Philippines	CHRB	ITS1, 5snts
Leea quadrifida Merr.	University of San Carlos 821	Surigao del Sur, Philippines	L	ITS1
Leea rubra Blume ex Spreng.	Lee 127	Singapore	SING	ITS, 5snts
Leea rubra Blume ex Spreng.	Martensz 718	Northern Territory, Australia	CANB	ITS1, 5snts
Leea rubra Blume ex Spreng.	Pullen 6703	Papua New Guinea	A	ITS1, 5snts
Leea setuligera C. B. Clarke	Chand 3311	Assam, India	MICH	5snts
Leea sp.	Bartlett 15924	Lanao, Philippines	MICH	ITS1
Leea sp.	Kress 37301	Myanmar	US	ITS, 5snts
Leea sp.	Kress 975877	Myanmar	US	ITS, 5snts
Leea sp.	Lorence and Lecordier 2647	Mauritius	MO	ITS
Leea sp.	Wen 8303	Mountain Prov., Philippines	US	ITS
Leea sp.	Yap s.n.	Palawan, Philippines	CHRB	ITS1, 5snts
Leea spinea Desc.	Barthelat 646	Mayotte	MO	ITS, 5snts
Leea zippeliana Miq.	Pullen 7351	Papua New Guinea	CANB	ITS1
Leea zippeliana Miq.	Schodde and Craven 4387	Papua New Guinea	CANB	ITS1, 5snts
Liquidambar orientalis Mill. (Saxifragaceae)	--	--	--	ITS1 (AF304524)
Tetrastigma sp. (Miq.) Planch. (Vitaceae)	Molina 4	Palanan, Philippines	CHRB	ITS

CHAPTER 2: Morphological evolution in the grape relative *Leea* (Leeaceae) with an updated species checklist

ABSTRACT

The current species circumscriptions (Ridsdale 1974, 1976) of *Leea* underestimate its genetic diversity, which may skew biodiversity estimates. In this chapter salient morphological characters were examined and reconstructed on the molecular phylogeny to understand *Leea's* morphological evolution. Chi-square tests were also performed to determine the associations of these characters with habitat types (grasslands/seasonally dry forests vs. rainforests; island vs. continental settings). Taxonomically discernible species are associated with island habitats and are mostly endemic. Members of clade II and III, predominantly distributed in the Malesian islands, exhibit synapomorphies such as spines (clade II) and significantly longer, thicker flowers (clade III). Within clade III, species from New Guinea are sister to a clade composed of Philippine species and both show complex endosperm rumination patterns. The Philippine species are unique in possessing four-merous flowers, while the New Guinean species bear significantly larger fruits (>20 mm). Cryptic morphotypes are associated with continental habitats, which could facilitate secondary contact between incipient species resulting in morphologically overlapping hybrids. This may have been the case for the Indian/Indochinese *L. asiatica* s.l. composing clade I, whose 'basal' phylogenetic position is supported by its retention of the plesiomorphic trait of free stamens. Flower color, which was used in the past to circumscribe the white-flowered (including greenish-

white and cream) *L. indica* from the red-flowered *L. guineensis,* was taxonomically unreliable and homoplastic, arising in multiple unrelated lineages within clade IV, in which other distinct morphospecies are also embedded. To resolve the polyphyly of species concepts within clade IV, cryptic entities were resurrected and tentatively assigned species names by comparing phylogenetically sampled vouchers with type images and protolog descriptions. These names were included in an updated tentative checklist of 47 species pending detailed taxonomic investigations that may reveal diagnostic characters. One new species from Northeast Philippines, *L. palanensis* sp. nov., and three putative new species from Myanmar and Palau are also recognized.

INTRODUCTION

Leea species are distinct from members of Vitaceae in not being viny, but rather display a monopodial erect habit, either as herbs, shrubs, or trees lacking tendrils, with terminal inflorescences, which in Vitaceae are often positioned opposite a leaf (Wen 2007a,b). They also possess a floral tube capped by fused stamens (Fig. 1), as well as large stipules that form a protective sheath around the developing leaves (Fig. 2). These morphological differences warrant segregation into its own monotypic family Leeaceae Dumort. (Planchon 1887; Ridsdale 1974, 1976; Latiff 2001; Wen 2007a).

Leea species grow in dry deciduous forests, open grasslands, or rainforests, throughout the old tropics, from Africa to Northeast Australia and the islands of the Pacific (Fiji, Solomon Islands, Caroline Islands), but are most diverse in Asia including India, Indochina (i.e. Myanmar, Thailand, Laos, Cambodia, Vietnam), and Malesia (i.e.

Malaysia, Singapore, Indonesia, Brunei, Philippines, New Guinea). Ridsdale's revisions (1974, 1976) documented about 34 species, but Li (1998) reported as many as 153. Clarke (1881) revised the Indian species of *Leea,* which amounted to 29 species in that region alone. He split the genus into two series: the red-flowered *Rubriflorae* and the green-flowered *Viridiflorae*. These were not adopted by Ridsdale (1976: 756), who wrote the most comprehensive monograph of the genus, since he found them "unreliable". Even Clarke himself admitted that he had "little confidence in the limits of any (species), except the Bengal ones" (Clarke 1881: 100).

The dilemma of uncertain species delineations may have also plagued Ridsdale (1974, 1976:756), declaring that "some species appear to be very variable." Leaf architecture is often variable within a species and cannot be used reliably for identification, as in the case of his circumscription for *Leea macrophylla,* which includes unifoliolate to 3-pinnate forms (Ridsdale 1974). In his revision of Malesian species, Ridsdale (1976) combined overlapping morphological entities, ranging from glabrous, small-leaved forms (c. 30 mm long) to pubescent, large-leaved morphs (c. 300 mm long) into species complexes, that encompass vast geographical distributions, like the red-flowered *L. guineensis* G. Don and the white-flowered (sometimes cream, pale yellow, greenish) *L. indica* (Burm. f.) Merr. (Molina, pers. obs.).

The recent molecular phylogenetic study for Leeaceae (Molina, chapter 1) has shown that Ridsdale's species concepts of *L. indica* and *L. guineensis* are polyphyletic and need to be revised. In this chapter, ancestral states of morphological characters, that have been used in the past for taxonomic diagnoses, are mapped on the molecular phylogeny to understand character evolution in *Leea.* Associations of these characters

with habitat types--islands vs. continental settings and rainforests vs. non-rainforests (savannahs/dry, seasonal forests)--are also conducted to determine the adaptive value of these traits and speculate on the mechanisms that underlie *Leea's* cryptic evolution (i.e. speciation not accompanied by morphological change, Bickford et al. 2007). Similar patterns of morphostasis have been reported in many other paleotropical plant taxa such as *Aglaia* (Meliaceae)*, Diospyros* (Ebenaceae, Pannell and White 1988), *Macaranga,* and *Mallotus* (Euphorbiaceae, Kulju et al. 2007). An overwhelming amount of these taxonomically frustrating transitional forms in plants exist in the geologically complex Malesian region (Hall 1998; Morley 2000; Woodruff 2003), which has provided not only opportunities for diversification but also the breakdown of incipient speciation, giving rise to a range of hybrids with intermediate morphologies.

Conservation priorities depend on species richness and levels of endemism, but the monograph of *Leea* (Ridsdale 1974, 1976) underestimates its genetic diversity. This trend may also be true for other Southeast Asian biota with undetected species, which seriously skews biodiversity estimates in this already highly threatened region. Thus, an updated checklist of *Leea* species is also presented in this paper based on the molecular phylogeny (Molina, chapter 1) and morphological features examined in this study.

MATERIALS AND METHODS

Herbarium specimens (c. 200) from A, CANB, F, K, L, MICH, MO, NY, UC, US (abbreviations following Holmgren and Holmgren 1998) were examined for morphological characters previously used for taxonomic diagnoses (Ridsdale 1974,

1976). Mesquite (Maddison and Maddison 2007) was used to infer ancestral states of nine discrete (Table 1) and 5 continuous traits [flower length (Fig. 1), leaflet length, margin width (i.e. width of each tooth of the serrate leaves), stipule scar: petiole length (Fig. 2E), and fruit diameter] on the Bayesian phylogeny (from BEAST analysis in chapter 4). For flower color, to avoid erroneous homology assumptions due to the subjective nuances for this trait, only corolla color (excluding color of floral tube, which is often white/cream; Molina, pers. obs.) was coded in Mesquite. In addition, collection notes describing the flower as greenish or pale-green, was also coded as white (0) since they were observed to be used interchangeably in different specimens of the same species (see discussion below).

Character history was traced using a parsimony criterion since likelihood reconstruction in Mesquite cannot handle polymorphic characters. To account for phylogenetic uncertainty, discrete ancestral states were summarized over the last 1000 trees generated by the Bayesian analysis in chapter 4 (i.e. BEAST analysis). This feature is currently unavailable for continuous characters, which were simply reconstructed on the consensus topology using the option "Trace character history" in Mesquite. The output reconstructions were summarized with synapomorphies mapped on the phylogeny (Fig. 3).

To determine habitat associations of morphological traits, chi-square (2x2 contingency) tests were performed by counting the number of morphospecies displaying a particular trait in 1) island vs. continental settings; 2) rainforests (primary or secondary) vs. non-rainforests (i.e. savannah or dry seasonal forests). Traits examined for correlations were: flower length (calyx base to floral tube, Fig. 1), fruit diameter, leaflet

length, and presence of hairs on leaves and petiole/petiolule. The first three continuous characters were made into categorical variables by determining the mean+95% CI of the measurements for entities sampled outside clade III, and values higher and lower than these (3.1 mm for flower length, 8.9 mm for fruit diameter, 162.8 mm for leaf length) were counted and tallied. Table 2 shows an example of the contingency table used for determining the association between flower length and habitat type. The number of endemic and cryptic morphospecies were also independently tallied against island or continental settings. All χ^2 values exceeding the critical value of 3.84 ($df=1$, $p < 0.05$) led to the rejection of the null hypothesis of equal distributions (i.e. no relationships).

Table 1. Discrete morphological characters reconstructed in Mesquite

Discrete trait	States		
Corolla color	White/cream/greenish-white (0)	Yellow-orange (1)	Red (2)
Endosperm rumination	Simple (0)	Complex (1)	--
Large glands on leaf underside	Absent (0)	Present (1)	--
Hairs on leaves and petiole/petiolule	Absent (0)	Present (1)	--
Hairs on inflorescence peduncle	Absent (0)	Present (1)	--
Flower merosity	4 petals (0)	5 petals (1)	--
Pinnation	Unifoliolate (0)	Once-pinnate (1)	Multi-pinnate (2)
Spines	Absent (0)	Present (1)	--
Stipule shape	Wing-type (0)	Obovate (1)	--

Table 2. Example of contingency table to determine if flower length was associated with island/continental habitats.

	>3.1 mm flower length	< 3.1 mm
Island	15	8
continental	2	9

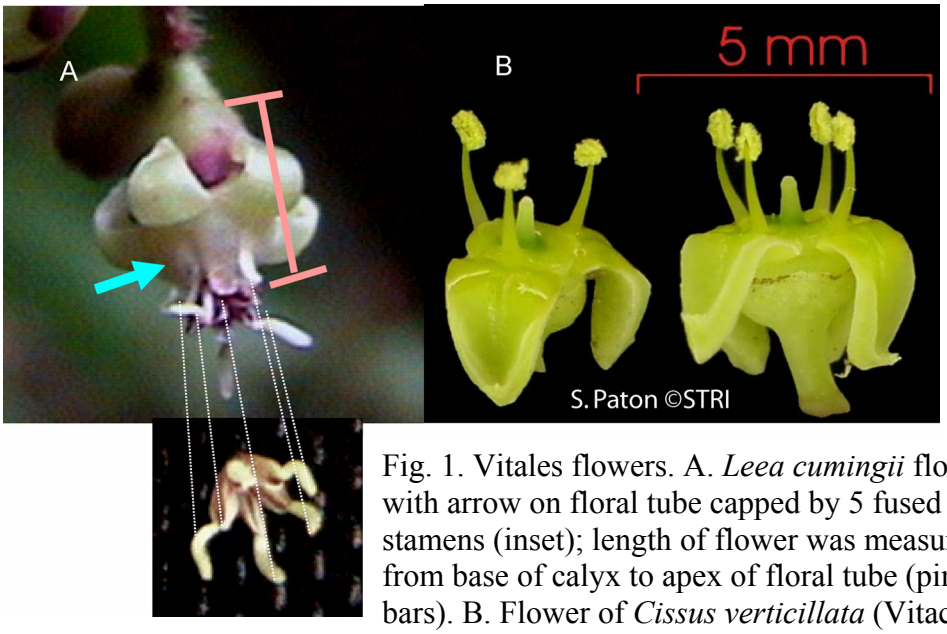

Fig. 1. Vitales flowers. A. *Leea cumingii* flower with arrow on floral tube capped by 5 fused stamens (inset); length of flower was measured from base of calyx to apex of floral tube (pink bars). B. Flower of *Cissus verticillata* (Vitaceae) lacking a floral tube and with free stamens.

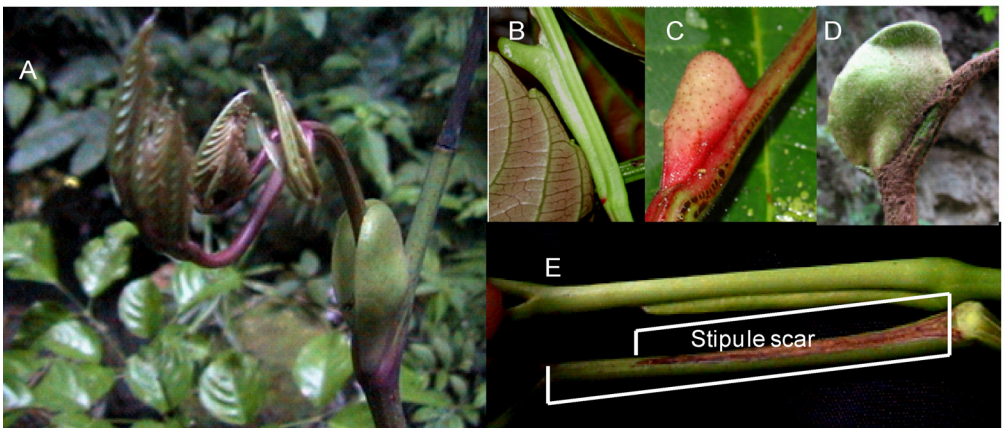

Fig. 2. A-E. *Leea* stipules. Stipule of *Leea negrosense* enclosing developing leaves. B-E. Variations in stipule morphology. B. *Leea aculeata*, wing-type stipule. C. *Leea palanensis* n. sp., obovate type. D. *Leea cumingii*, obovate type. E. *Leea philippinensis,* wing-type (top); stipule scar and petiole length ratio (SC:PT), bottom.

RESULTS AND DISCUSSION

Character evolution

A few characters were strong synapomorphies for certain clades such as the spines for species in clade II, four-merosity for the Philippine species of Clade III, and the discoid large glands on the underside of the leaves for *L. aequata.* Clade I also has free stamens, a feature not seen in any other *Leea* species, but is a plesiomorphy shared with Vitaceae. However, aside from molecular features (Molina, chapter 1), it was difficult to pinpoint gross morphological synapomorphies uniting each of the species within clades I or IV, or between II+III and IV. The lack of seeds and fruits in most specimens precluded detailed studies of these characters, but based on mapping Ridsdale's descriptions on the phylogeny, complex endosperm rumination evolved in the ancestor of the New Guinea and Philippine species of clade III (Fig. 3). This clade is also composed of species that possess about 32% longer leaves and 36% longer, thicker (coriaceous) flowers and 50% larger fruits (Fig. 3) than those found in other clades (I, II, IV). However, the evolution of longer flowers may not be strictly confined to clade III, as these also occurs in other island taxa such as *L. tinctoria* (São Tomé) and *L. grandifolia* (Nicobar and Andaman Islands) that may not be closely related to members of clade III, though this needs to be phylogenetically tested.

Flower and fruit size evolution. Longer flower size (>3.1 mm long) was significantly associated with island habitats (χ^2=6.58, df=1) but this was not true for fruit size (χ^2=3.13, df=1). However, the strength of the relationship between flower size and island habitats actually arises from the diversity of species within clade III (13/23 island

spp.) exhibiting this feature. The correlation thus appears to be an artifact of phylogenetic niche conservatism, wherein the large-flowered ancestor of clade III failed to diversify beyond island settings. Nonetheless, this feature presents as a strong synapomorphy for this clade (Fig. 3).

Lendvai and Levin (2003) reported that the evolution of larger floral size in *Phlox* (Polemoniaceae) might have resulted from changes in the ancestral pollinator assemblage with the larger size perhaps selected for by more efficient pollinator service. They argued that a decrease in floral size may have been due to the decrease in pollinators encouraging higher levels of selfing. As for *Leea*, the ancestor of clade III may have encountered an abundance of potential pollinators when it first reached Malesia prompting floral size changes to cater to the new but more competent pollinators.

Though many species in clade III have relatively larger fruits, this is not correlated with their island habitat, with the New Guinean species possessing the largest fruits. The evolution of bigger fruits could have been influenced by the different assemblage of frugivore dispersers. Pannell and White (1988) discussed the importance of understanding the ecology of dispersal to resolve species complexes, which usually vary in fruit morphology to accommodate the different kinds of dispersers. Unfortunately, this information is often missing in herbarium specimens. As an example, they noted that the absence of typical avian and primate dispersers of the tropical plant *Aglaia* in East Malesia had been compensated for by ground-dwelling birds (cassowary) and rodents, which preferentially feed on fallen fruits. Hence, it makes sense that the New Guinean endemics of clade III had evolved the largest fruits in the genus perhaps to facilitate their descent to the ground after abscission.

Flower color evolution. Flower color is believed to be the most important feature distinguishing the red-flowered *L. guineensis* from the white-flowered *L. indica* (Ridsdale 1976). Though *Leea guineensis* was originally described by G. Don in 1831 after a species native to New Guinea with white/greenish-white flowers, Ridsdale (1974, 1976) had redefined it to represent red-flowering morphospecies. Following Ridsdale's (1976) treatment, it is impossible to tell *L. indica* s.l. and *L. guineensis* s.l. apart without flower color information, which he may have personally experienced when he inadvertently identified non-flowering duplicates of the *Fenix 24980* collection from Mindanao, Philippines, deposited in US and UC, as being *L. indica* and *L. guineensis*. Ridsdale (1976:778) admitted that his circumscriptions make them species complexes that encompass a "wide range of variability, both geographically and ecologically". Phylogenetic analysis using DNA sequences confirmed this and showed the polyphyly of *L. guineensis* and *L. indica* in chapter 1.

It must be clarified that flower color is a composite of corolla lobe color and floral tube color, which are often different. For example, *Leea aculeata* has a white floral tube surrounded by light green petals while *L. luzoniensis* Elmer (included in *L. guineensis* by Ridsdale 1976) has red petals enclosing a white floral tube (Fig. 3). The color of the calyx, which is the first floral structure to develop since it envelops the flower bud, also does not necessarily translate to petal color, such that in *L. rubra* the bright red calyx subtends the pale-orange petals and whitish floral tube (*Pullen 6703; Specht 1305*). This may not have been realized by Ridsdale (1974, 1976) as some herbarium sheets noted flower color as "red buds", which he then assumed to be *L. guineensis*. One

morphospecies of *L. guineensis* s.l. (=*L. cumingii* Clarke) in the Philippines possessed white petals, but the calyx enclosing floral buds are tinged with red (Molina, pers. obs.).

Coding color in herbarium sheets is also prone to subjectivity, with collection notes recording white to cream to greenish white for the corolla of the generally white-flowering species, while the supposedly reddish-petaled species are described with a range of colors from pink to bright orange to crimson to maroon. These color differences were actually seen in various collections for the same species (Molina, pers. obs.), such as in *L. rubra,* wherein flower color was independently noted as: "maroon", "red calyx, pale orange petals", "creamy white", and "red corolla, cream inside, corolla and stamens pale cream", or in *L. philippinensis,* where color was reported as: "green and white", "white", "pale yellow", "cream", "flowers pink with green petals". Such variations in flower color also lend support to the lability of this trait and its unreliability in circumscribing *Leea* species.

White (including greenish-white and cream) corolla color (Fig. 3) was reconstructed to be the ancestral state. Petal color is homoplastic, with reddish petals showing up in unrelated lineages of clade "d", suggesting the color's adaptive value, perhaps in response to changes in pollinator assemblage in new environments. Yellow-orange petals have evolved in subclade "c", which comprise the Indochinese/African/ Madagascan entities. Due to a change in pollinators, change in pigmentation can lead to genetic isolation, and ultimately to speciation (Durbin et al. 2003). Hence, the entities comprising clade IV need to be re-circumscribed to account for the several morphospecies comprising this diverse clade.

Leaf evolution. Leaf length (χ^2=1.71, df=1) was not significantly associated with either island or continental settings. Though leaf margin width (i.e. width of each tooth) was observed to be variable among specimens (and uncorrelated with leaf width), with *L. asiatica* developing extremely serrate (c. >1.5 mm margin width) margins, it was not taxonomically useful especially for entities in clade IV.

Clades I, II and III all exhibit 1-pinnate leaves (rarely unifoliolate), whereas clade IV is predominantly at least twice-pinnate, with very few exceptions, such as subclade "b" which includes both unifoliolate (*L. macrophylla* s. str.) and pinnate forms (*L. robusta*; Wen, pers. comm.). The *Leea* ancestor is predicted to be once-pinnate. Clade IV shares the synapomorphy of multi-pinnate leaves that may have conferred some form of evolutionary advantage to the members of this clade as exemplified in its diversity compared to other clades. In the past, leaf morphology was considered to be taxonomically important, which led to a proliferation of species being recognized (Wen 2007a), but Ridsdale believed that degree of leaf pinnation varies depending on ecological conditions. However, this does not apply to members of clades I, II and III, which do not display multi-pinnate leaves in spite of varying ecological settings. Gerrath and Lacroix (1997) have shown that in *L. guineensis,* leaf pinnation is correlated with ontogeny, with the first few nodes developing simple leaves while successive nodes forming increasingly pinnate leaves. Hence, degree of pinnation is not generally a useful taxonomic character.

Pubescence. Mapping degree of vegetative pubescence (petiole/petiolule and leaves) on the phylogeny reveals that this trait is convergent appearing in divergent lineages. The chi-square test (χ^2=7.64, df=1) shows that vegetative pubescence is

significantly linked with habitat type, with drier/seasonal habitats housing pubescent forms, while rainforests (primary/secondary) supporting glabrous morphs. Subclade "d", which includes taxa surviving in both habitats, demonstrates the plasticity of this trait. For example, the species, *L. guineensis* s. str. (New Guinea, Australia, Pacific Islands), previously included in *L. indica*, has both glabrous *(Regalado 705)* and pubescent forms (*Takeuchi 4320*). A study by Ehleringer et al. (1981) reported the adaptive potential of leaf pubescence, increasing in plants growing in arid habitats versus those growing in moist conditions. Pubescence provides protection against excessive insolation, heat, herbivory, and water loss. However, inflorescence pubescence in *Leea* does not show the same pattern of parallel evolution, but may be phylogenetically constrained, at least for the members of clade IV, whose members all possess hairy peduncles, irrespective of a dry or wet habitat type.

Stipule evolution. Ridsdale emphasized the diagnostic value of stipule shape in species identifications and provided detailed illustrations of these in his revisions, with those of *L. indica* and *L. guineensis* encompassing a variety of stipule morphologies. However, these cannot be directly assessed on herbarium specimens since most of them were deciduous, but the ratio of stipule scar length to petiole length (SC:PT, Fig. 2E) is directly measurable. A stipule scar measuring ≥0.5 of the entire length of the petiole is presumably the ancestral condition with clades I, II, III (except *L. amabilis, L. congesta, L. coryphantha, L. heterodoxa*) possessing this type. Most often an SC:PT ≥0.5 refers to a narrow wing-type stipule (Fig. 2B, E vs. short-obovate: Fig. 2A, C, D) but exceptions occur, such as *L. amabilis* which has a ratio of 0.4 but has a wing-type (Ridsdale 1974). SC:PT ≥0.9 appears to be a synapomorphy for clade I, while clade II has an SC:PT of at

least 0.7. Clade IV members, on the other hand, exhibit shorter stipule scars relative to the petiole (<0.5) and this is also often associated with the obovate-type, but *L. macrophylla's* s. str. obovate stipule is an exception since its scar ratio is 0.7. It is unclear what could have triggered such evolutionary variations in stipule length, but it seems likely that this change was not dictated by habitat type since both forms occur in either rainforest or open, dry, and seasonal habitats.

Cryptic speciation and its taxonomic implications

Based on the patterns of character evolution presented above, *Leea* had undergone cryptic evolution or morphostasis, with the exception of members comprising clades II and IV, which are readily identifiable by the spines and larger floral/fruit structures, respectively. Species comprising these clades are also taxonomically discernible, as opposed to many of the morphospecies composing clades I and IV. It is worth noting that the readily identifiable species (non-cryptic) are often associated with islands (χ^2=6.13, df=1), whereas the cryptic forms are found in continental habitats (Indochina). Endemics are also shown to be linked with island habitats (χ^2=12.74, df=1), which is understandable, since it is more difficult to traverse intervening seaways. In a continental setting it is easier for secondary contact between incipient species to occur. Thus, cryptic species may develop through hybridization or through active speciation of metapopulations resulting from fragmentation of an originally contiguous ancestral population (Struwe, pers. comm.). Species circumscriptions become difficult in this case since overlapping morphological entities can only be distinguished by molecular

characters. This may be true for clade I and most members of clade IV (Molina, chapter 1).

Leea asiatica, as circumscribed by Ridsdale (1974, 1980), may be a species complex composed of two distinct morphotypes: *L. crispa* and *L. aspera* (Molina, chapter 1). *Leea asiatica* s. str. (=*L. crispa* sensu Lawson 1875) is a polyploid (2n=48). It is characterized by petioles and peduncles with crisped wings and acutely serrate leaves that are glabrous above. On the other hand, *L. aspera* (sensu Lawson 1875) displays the common ploidy level of 2n=24. It differs from *L. asiatica* s. str. in having leaves with white scattered appressed hairs between nerves and generally crenate-serrate margin (Lawson 1875). I have personally seen intermediates, probably hybrids, which may have prompted Ridsdale (1974) to combine these entities. Detailed cytological and phylogeographic work are needed to resolve this species complex.

Ridsdale (1974) also broadly circumscribed *L. macrophylla* to include four entities with a variety of habits--herbs, shrubs, or trees--exhibiting increasing degrees of pubescence and pinnation, from unifoliolate (*L. macrophylla* s. str.) to 3-pinnate. His decision to combine all these entities was justified by his observation of an essentially uniform floral structure and their sympatric distributions pending detailed ecological investigations supporting taxonomic separation. Mathew and Umadevi (1992) argued that *L. macrophylla* is distinct from *L. robusta* as the former is not known to produce pinnate forms. In the following checklist, this treatment is also followed.

The lack of any discernible gross morphological feature in many of the entities included in clade IV has repeatedly confounded taxonomic circumscriptions. This cryptic evolution underreports the genetic diversity inherent in the clade (Molina, chapter 1).

Morphospecies that are diagnosable such as *Leea aequata, L. rubra, L. setuligera, L. compactiflora,* and *L. macrophylla* s. str. are also embedded in this clade. *Leea longifoliola,* Merr.*,* represented by *CH78319* in chapter 1, has been combined by Ridsdale (1976) with *L. indica,* though its leaflets are unique in being almost 5x longer than wide, which does not occur in any other *Leea* species. Other species, that were not phylogenetically sampled, but possibly included in clade IV are *L. alata, L. grandifolia, L. gonioptera, L simplicifolia, L. thorelii,* and *L. tinctoria* as they lack any of the observable traits displayed by either clades I, II, and III. It is imperative to recognize the molecularly-variable cryptic entities that have long been buried in the *L. indica/guineensis* complex as individual species.

Wen (pers. comm) believes that the notion of cryptic species, at least for plants, is an artifact of poor taxonomic investigation, such that the suite of morphological characters (e.g. anatomical) that can potentially provide specific diagnoses remains to be discovered. Pannell and White (1988) also argued that knowledge of fruit morphology and dispersal ecology are key in disentangling species complexes. As mentioned previously, flower color also plays crucial role in species evolution since it can alter the pollinator assemblage, which may ultimately lead to reproductive isolation. This is also tested empirically in chapter 3 for three sympatric morphospecies within a primary forest plot in Northeast Philippines, which is home to at least six *Leea* entities. *Leea* morphospecies vary considerably in their habit (herbs, shrubs, or trees) and habitat ecology (riverine forest, inland forest, open secondary vegetation), which may be taxonomically important characteristics, that have often been lacking from herbarium collection notes (Molina, pers. obs.). Wen (pers. comm.) also observed these ecological

variations in her field trips to Madagascar and Thailand, which still have undescribed *Leea* morphospecies.

Based on morphological and ecological observations in the Philippines, a new morphospecies is reported here, *Leea palanensis* sp. nov., that would have otherwise been assigned to *L. indica* by virtue of its white corolla. Though they cluster together in the molecular phylogeny (Fig. 3), they vary significantly morphologically. *Leea palanensis* exhibits a short stature (<1 m, suffrutuscent herb), a smaller glabrous stipule (Fig. 2C), and glabrous leaves, which contrast with those of the sympatric *L. cumingii* (combined with *L. guineensis* s.l. by Ridsdale, 1976), which is a small tree (3-6 m) and is extremely pubescent. The calyx of *L. palanensis* is pale green and does not exhibit the reddish tinge characteristic of *L. cumingii.* Such detailed flower color, which may represent an important evolutionary trait, is often missing from herbarium notes.

As morphological variations between species are more conspicuous in their natural habitat, revisionary studies of *Leea,* or of any other taxonomically challenging group, must be supplemented with detailed color studies of reproductive structures and information on habit and pollination/dispersal ecology. Pannell and White (1988: 651) asserted that "biological understanding is ultimately more important than the precise way in which facts are slotted into a rigid formal taxonomy." Since conservation priorities rely on biodiversity estimates, it is critical that species circumscriptions reflect the historical processes that have influenced modern genetic diversity. An updated tentative species checklist for *Leea,* including resurrected (11 spp., i.e. cryptic species) and new species (4 spp.), is included here based on the molecular synapomorphies supporting clades (Molina, chapter 1), in spite of the lack of discernible morphological features in the hope

that future detailed taxonomic and ecological work will reveal the characters supporting these species concepts. Names for resurrected species were based on protolog descriptions and morphological similarity of the types with the specimens sampled for phylogenetic analyses in the first chapter.

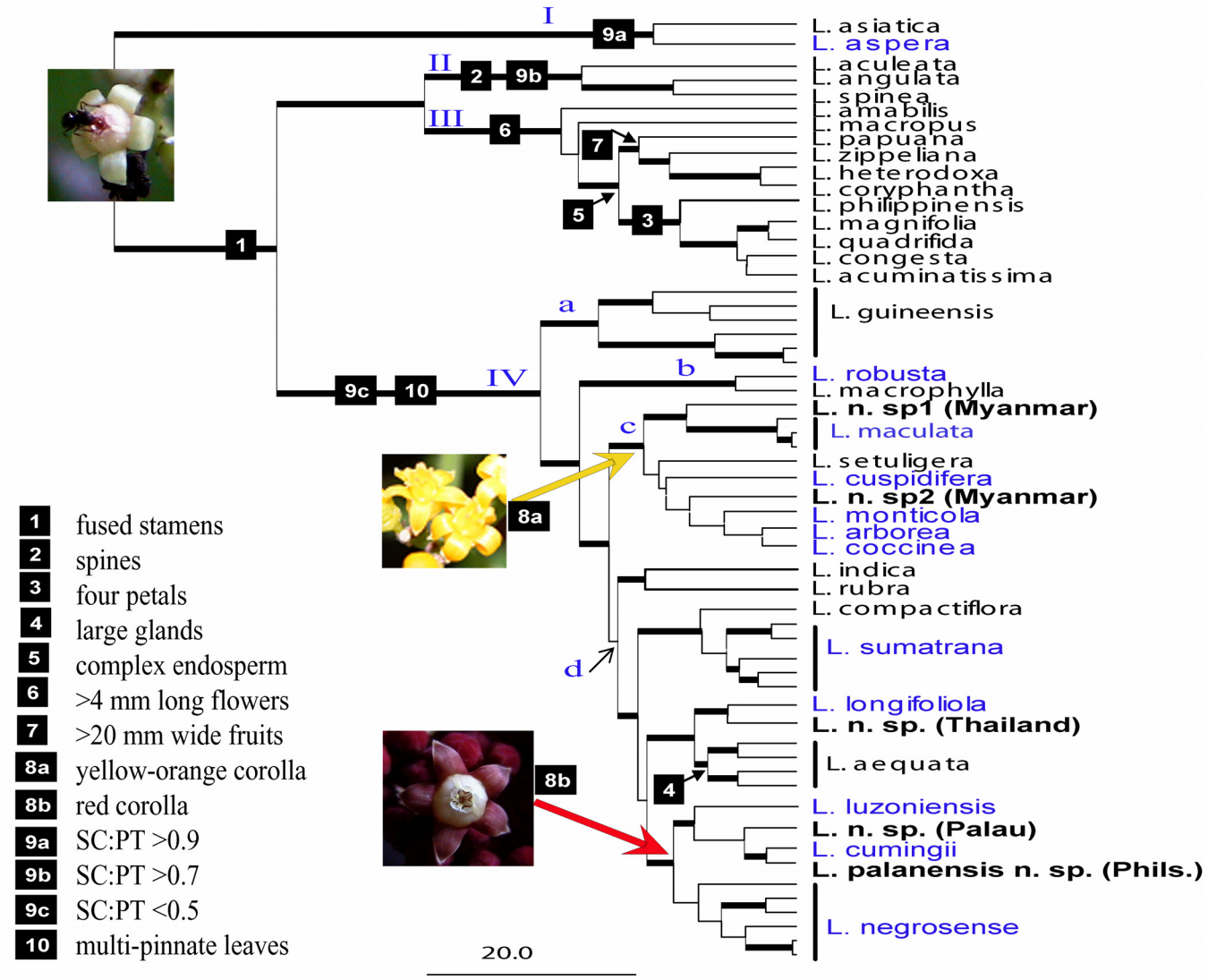

Fig. 3. Morphological synapomorphies (1-10) mapped onto the Bayesian phylogram (from BEAST analysis in chapter 4). Bold horizontal lines represent clades with ≥90% posterior probabilities. Taxon names reflect species names in the updated checklist (Table 3). Resurrected species names are in blue and putative new species are in bold. The ancestral state for flower color is white/ greenish/cream (top left). Major clades (I-IV) and subclades (a-d) are labeled as in chapter 1.

Table 3. Updated checklist of *Leea* species. Resurrected names are marked with *. Putative new species are in bold. Specific identities of *L. curtisii, L. krukoffiana, L. saxatilis, L. smithii* are considered doubtful pending further taxonomic investigation and are not included here.

Taxon names	Geographic distribution
1. *Leea aculeata* Bl. ex Spreng.	Sumatra, Java, Borneo, Philippines, Sulawesi
2. *Leea acuminatissima* Merr.	Philippines
3. *Leea aequata* L.	India, Bhutan, Nepal, Bangladesh, Andaman Islands, Myanmar, Thailand, Cambodia, Laos, Vietnam, Malaya, Singapore, Sumatra, Java, Lesser Sunda Islands, Borneo, Philippines, Sulawesi, Moluccas
4. *Leea alata* Edgeworth	India, Nepal, Bhutan
5. *Leea amabilis* Veitch	Borneo
6. *Leea angulata* Korth ex Miq.	Nicobar Islands, Thailand, Malaya, Singapore, Sumatra, Java, Lesser Sunda Islands, Borneo, Sulawesi, Moluccas
7. **Leea arborea* Sieber ex Bojer	Mauritius
8. *Leea asiatica* (L.) Ridsdale	India, Bhutan, Nepal, Bangladesh, Andaman Islands, Myanmar, Thailand, Cambodia, Laos, Vietnam
9. **Leea aspera* Wall.	India, Bhutan, Nepal, Bangladesh, Myanmar
10. *Leea compactiflora* Kurz	India, Bhutan, Myanmar, Laos, Vietnam, tropical China
11. **Leea coccinea* Planch.	Myanmar
12. *Leea congesta* Elm.	Philippines
13. *Leea coryphantha* Laut.	New Guinea
14. **Leea cumingii* Clarke	Philippines
15. **Leea cuspidifera* Baker	Madagascar
16. *Leea gonioptera* Laut.	New Guinea
17. *Leea grandifolia* Kurz	Andaman and Nicobar Islands
18. *Leea guineensis* G. Don	New Guinea, Australia, Fiji, Solomon Islands
19. *Leea heterodoxa* K. Sch. & Laut.	New Guinea
20. *Leea indica* (Burm. f.) Merr.	India
21. **Leea longifoliola* Merr.	Tropical China, Myanmar
22. **Leea luzoniensis* Elmer	Philippines
23. *Leea macrophylla* Roxb. ex Hornem.	India, Bhutan, Nepal, Bangladesh, Andaman Islands, Myanmar, Thailand, Cambodia, Laos, Vietnam
24. *Leea macropus* K. Sch. & Laut.	Bismarck Archipelago
25. **Leea maculata* Desf.	Tropical Africa
26. *Leea magnifolia* Merr.	Philippines
27. **Leea monticola* Desc.	Madagascar
28. **Leea negrosense* Elmer	Philippines
29. ***Leea palanensis* n. sp.**	Philippines

Table 3 (cont'd.)

30. *Leea papuana* Merr. & Perry	New Guinea
31. *Leea philippinensis* Merr.	Philippines, Taiwan
32. *Leea quadrifida* Merr.	Philippines
33. **Leea robusta* Roxb.	India, Myanmar?, Thailand, Cambodia?, Laos?, Vietnam?
34. *Leea rubra* Bl. ex Spreng.	India, Bangladesh, Myanmar, Thailand, Cambodia, Laos, Vietnam, Malaya, Singapore, Sumatra, Java, Lesser Sunda Islands, Borneo, Philippines, Sulawesi, Moluccas, New Guinea, tropical Australia
35. *Leea setuligera* Clarke	India, Thailand, tropical China
36. *Leea simplicifolia* Zoll & Mor.	Thailand, Malaya, Java
37. *Leea spinea* Desc.	Madagascar, Comoros
38. *Leea sumatrana* Miq.	Malaya, Sumatra, Singapore, Borneo?, Philippines, Java?, Sulawesi
39. *Leea tinctoria* Baker	São Tomé
40. *Leea tetramera* Burtt	Solomon Islands
41. *Leea thorelii* Gagnep.	Thailand, Cambodia, Laos, Vietnam
42. *Leea unifoliata* Merr.	Philippines
43. *Leea zippeliana* Miq.	New Guinea
44. ***Leea* n. sp.1**	Myanmar
45. ***Leea* n. sp.2**	Myanmar
46. ***Leea* n. sp.3**	Palau (Caroline Islands)
47. ***Leea* n. sp.4**	Thailand

CHAPTER 3: Floral biology of Philippine morphospecies of the grape relative *Leea* (Leeaceae)

ABSTRACT

I observed the floral biology of three *Leea* morphospecies in a Philippine natural forest habitat. The red-flowered morphospecies *Leea guineensis* limits selfing through synchronized dichogamy, with male and female flowers temporally separated in the same inflorescence, while the two morphospecies of the white-flowered *L. indica* may be prone to geitonogamous selfing. Light and soil pH are correlated with phenology. In addition to bees and flies, *Leea* is visited by wasps, butterflies, beetles, bugs, and spiders.
Key words: dichogamy; geitonogamy; phenology; Philippines; pollination

INTRODUCTION

The genus *Leea* (Leeaceae) is the closest relative to the economically important grape family, Vitaceae (Ingrouille et al. 2002; Soejima and Wen 2006; Wen et al. 2007a, b), but unlike Vitaceae, *Leea* species are erect shrubs or trees lacking tendrils, with flowers showing a distinct floral tube. In spite of *Leea's* evolutionary affinity with the grapes, its systematics and floral ecology are poorly known. The latest revision (Ridsdale 1974; 1976) reported 34 species distributed in the tropics of Africa and Asia, where the genus is most diverse. However, recent phylogenetic studies using nuclear markers show that two species, *L. indica* and *L. guineensis,* which are the subject of the present study,

are polyphyletic species complexes that include narrowly-distributed endemic cryptic species (J. Molina, in prep.).

Like some members of Vitaceae (Gerrath et al. 2004; J. Gerrath, pers. comm.), *Leea* flowers are markedly protandrous (Gerrath et al. 1990). Though protandry may limit opportunities for pollen-stigma interference (Lloyd and Webb 1986) and autogamy (*i.e.*, same-flower selfing) that may cause inbreeding depression (Charlesworth et al. 1987), it cannot ensure that geitonogamy (i.e. selfing between flowers in the same inflorescence, or between inflorescences of the same plant) will not occur (Hessing 1988; Bhardwaj and Eckert. 2001). *Leea* species are bisexual and bear inflorescences with simultaneously blooming flowers that are possibly self-compatible (J. Molina, unpubl.), making them susceptible to geitonogamy (Hessing 1988). Geitonogamy may cause inbreeding depression (Routley and Husband 2003), spontaneous abortions and decreased fruit set (Finer et al. 2003), as well as pollen discounting (Harder and Wilson 1998), which reduces the amount of pollen available for outcrossing.

Geitonogamy is thus an important force in influencing the evolution of floral display (Routley and Husband 2003; Barrett 2003). Unless dichogamy is synchronized such that all male-phase flowers are temporally segregated from all female-phase flowers within an inflorescence or between inflorescences of the same plant, then geitonogamy could reduce reproductive success (Snow et al. 1996; Bhardwaj and Eckert 2001).

This study explores how Philippine morphospecies of *Leea indica* sensu Ridsdale and *L. guineensis* sensu Ridsdale may limit autogamy and geitonogamy in a natural population. The existence of a strategy for synchronized dichogamy was tested. Environmental correlates of phenology were also assessed to understand the factors that

influence flowering. Insect visitation was observed since pollinating species were unknown. Here, I elucidate *Leea's* floral biology, which may provide insights on the evolutionary significance of its floral traits.

MATERIALS AND METHODS

Field work was conducted from June to November 2004 and 2005 and July to August 2007 in the Palanan Forest Dynamics Plot, a 16-ha tropical research site located in northeast Philippines, on the island of Luzon (17°02' 36" N, 122°22'58" E). Flowering of the two morphospecies of the white-flowered *L. indica* (Burm.f.) Merr. (glabrous and hairy vegetative forms) and of the red-flowered species, *L. guineensis* G. Don were monitored. Immature inflorescences (*i.e.*, buds only) were tagged. *Leea guineensis* and the hairy *L. indica* are small trees (*ca* 3-6 m high), while the glabrous *L. indica* is a small clonal shrub (< 1 m high).

Flower development. Flowering stages (*i.e.*, buds: stage 1 bud <4 mm in length, stage 2 bud ≥4 mm; open flowers: stages 3 and 4 as male-phase, stages 5 and 6 as female-phase; see Results for details) were differentiated by visual characterization of morphological changes occurring during flowering development. To determine the mean time spent on each stage, development of 22 hairy *L. indica* stage 2 buds were observed from 0840 h to 1700 h for two consecutive days, noting the stage as they developed. Stigma receptivity was tested by applying hydrogen peroxide directly to the stigma (Dafni 1992; Kearns and Inouye 1993). The entire gynoecium was removed from putative female-phase flowers and inserted within a capillary tube. Hydrogen peroxide

was injected through the other end of the tube. Bubble formation on the stigma indicated receptivity (Dafni 1992). Style length (SL) of stages 1 to 6 was measured (N=18).

Synchronized dichogamy. For each morphospecies three inflorescences, which produced 85-233 buds (sum of stages 1, 2) in hairy *L. indica*, 65-177 buds in the glabrous *L. indica*, and 779-1600 buds in *L. guineensis* were analyzed for this study. Counts of stages were generally done between 0800 h to 1400 h once daily. To test for synchronized dichogamy, the percentage of open flowers experiencing no overlap between male-phase and female-phase (PNO) was compared with null expectations using simulations in R version 2.6.1 (The R Foundation for Statistical Computing, 2007). On each simulation I generated a number of open female flowers for each inflorescence. This number was an integer uniformly distributed between 0 and the total number of open flowers for that inflorescence. The number of male flowers was taken to be the difference between the total observed number of flowers and the simulated number of female flowers for each inflorescence. The percent of inflorescences with either zero male or female flowers was calculated for each simulation. The experiment was repeated 1000 times. If no-overlap between the number of male and female flowers is a random event, then the simulated PNO's should be similar to the observed PNO. If the observed PNO is found to be in the tail of the empirical distribution function of the simulated PNO's then there is evidence that the observed PNO is different from that which would be expected if no-overlap between the number of male and female flowers occurred randomly. For this experiment, I chose an alpha level of 5%, and concluded that this phenomenon was not random if the observed PNO was greater than 95% of the simulated PNO's (adopted from Bhardwaj and Eckert 2001).

Autonomous autogamy. To test for spontaneous self-pollination, insects were excluded by bagging immature inflorescences of four individuals per *Leea* morphospecies in contrast to a control exposed to open pollination. Total number of buds (sum of stages 1, 2) observed were 46-220, 52-162, and 365-2581 for hairy *L. indica,* glabrous *L. indica,* and *L. guineensis,* respectively. Fruit set was determined by dividing the total number of fruits by the highest bud count recorded for the duration of flowering. Nested ANOVA was performed to compare fruit set in both bagged and unbagged treatments.

Environmental correlates. Ambient light, moisture, and soil pH levels were measured by inserting the probes of the Sunleaves® three-way meter into the soil surrounding the plant to be tested. The meter provides rough estimates of these three factors that affect plant growth. Light readings run from a scale of 0 (low light) to 2000 (very strong light), pH readings run from pH 0 (acidic) to pH 10 (basic), and moisture readings run from a scale of 0 (dry) to 10 (moist). Ambient temperature was also measured. Only the hairy *L. indica* was tested. Linear regression was performed to determine how the stages of flower development are affected by environmental cues.

Insect visitation. Frequency of insect visitation was monitored by tallying the number of visitors that landed on the reproductive parts of a particular stage for at least 3 seconds within 1.5 h (between 0900 h-1030 h and 1500 h- 1630 h) for 10 observation periods for each *Leea* morphospecies. Visitation was inspected in the morning and afternoon to avoid possible temporal preferences of the flowering stages (e.g. male stages being dominant in the morning and/or female stages being dominant in the afternoon). Visitors were collected for taxonomic identification but not examined for pollen. One-

way ANOVA was performed to compare visitation frequencies among flowering stages for each *Leea* morphospecies. Insect visitation frequencies were compared among the three *Leea* morphospecies with two-way ANOVA.

RESULTS

Flower development. *Leea* flowers abundantly for about three months, June-August for both forms of *Leea indica* and August-October for *L. guineensis*. Individual flower development was specifically noted for the hairy *L. indica*, which produces flowers with two-day life spans. Six flowering phases were observed (Fig. 1), starting from small buds (stage 1, Fig. 1A) until senescence (stages 6-7, Fig. 1, N-O). Stage 2 buds (Fig. 1A-C) are at least 4 mm in length, bigger than stage 1 buds, and are about to open. Open flowers begin with stage 3 and last until stage 6. Stage 3 (Fig. 1D-E), which lasts about 27 min (N=27), marks the beginning of anthesis and of the male phase. Filaments hook over the floral tube and have not yet reflexed. Self-pollen is not yet presented, but insect visitors already probe the flower at this stage. This insect behavior seems to accelerate pollen presentation (J. Molina, pers. obs.).

Stage 4 begins (Fig. 1F-K) once the reddish-pink anthers are exposed and have dehisced. At this stage anthers form a staminal plug on top of the floral tube concealing the gynoecium. Anthers are initially laterally connivent and appear introrse (stage 4a, Fig. 1F-G), which is similar to Gerrath et al.'s (1990) greenhouse studies. After about 21 min

(N=22), the filaments recurve pulling the dehisced anthers until they are exserted (stage 4b, Fig. 1H), exposing the pollen. This stage lasts for about an hour (N=27), until the filaments begin to abscise basally (stage 4c, Fig. 1I-K) for the next 20 min (N=27). Stages 3 and 4 are considered male-phase and in total lasts for about 2 h.

For flowers of the hairy *L. indica* exposed to open pollination, female phase begins when the discoid stigma is already sufficiently exposed (stage 5, Fig. 1L-M). Stage 6 (Fig. 1N) represents the beginning of senescence with browning of the apices of the floral tube. Stages 5 and 6 are female-phase, which tested positive for stigma receptivity (N=6). Female phase is the longest, ensuing until the next day.

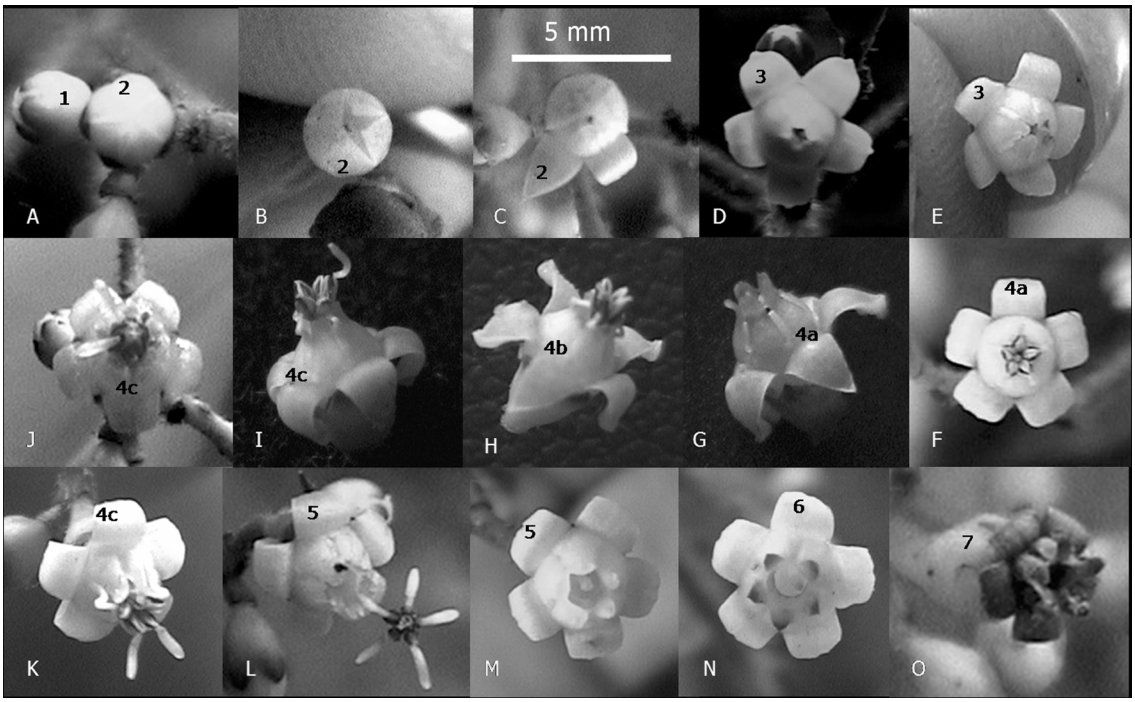

Fig. 1. Flowering stages in an individual flower of the hairy *Leea indica*. Numbers represent stages. (A) Stage 1, small bud *ca* 3 mm (left); (A-C) stage 2, large bud *ca* 4 mm; (D-E) stage 3, newly opened flower, anthers not yet visible; (F-K) stage 4, dehisced anthers visible (4a: filaments not yet reflexed; 4b: filaments have reflexed; 4c: filaments gradually detaching); (L-M) stage 5: stigma already visible; (N-O) stage 6, 7: senescence. Stages 3 and 4 are male-phase. Stages 5 and 6 are female-phase.

Flower development includes lengthening of the style. The mean style length (SL) for buds (stages 1, 2) was 0.9 ± 0.1 mm (mean ± 95% CI, N=4); male-phase (stages 3, 4), 1.2 ± 0.2 mm (N=8); and female-phase (stages 5, 6), 1.9 ± 0.2 mm (N=6). Mean SL of female-phase flowers were significantly different from mean SL of male-phase flowers (t-test, t-stat = -5.75, df = 11, $P < 0.0001$).

Synchronized dichogamy. Of the three morphospecies, none displayed statistically significant synchronized dichogamy, such that the observed PNO (percentage of open flowers experiencing no overlap between male-phase and female-phase) was greater than 95% of the simulated PNO's. *Leea guineensis* had some synchronized dichogamy, with its observed PNO of 66% greater than 91% of the simulated PNO's. The proportion of male and female flowers for both forms of *L. indica* were not significantly different from null expectations.

Autonomous autogamy. There was a significant difference in fruit set between bagged and unbagged treatments (Table 1; Table 2, nested ANOVA, $P < 0.0006$). There was no significant difference among fruit counts across the individuals tested ($P < 0.292$).

Environmental correlates. Environmental correlates for the hairy *L. indica* showed that total bud counts (sum of stages 1 and 2) were negatively correlated with soil pH ($P < 0.001$, adjusted $R^2 = 6\%$, N=180). The number of flowers in anthesis was positively affected by light ($P < 0.011$, adj. $R^2 = 7\%$, N=180). However, light and soil pH were not robust predictors in any of the regression equations yielding weak correlations. Soil moisture and temperature did not significantly influence flowering. The average for the four environmental conditions measured in three individuals of the hairy *L. indica* throughout its phenology were 350±40 for light intensity (mean ± 95% CI); 8.77±0.20 for

moisture; 7.15±0.05 for soil pH; and 29.08±0.21 ^{o}C for ambient temperature. It must be noted that measurements for light intensity and soil moisture are more qualitative because of the device used.

Table 1. Average fruit set percentage (total number of fruits divided by the highest bud count recorded) in bagged and unbagged treatments for the three *Leea* morphospecies

	Leea indica (glabrous)	*Leea indica* (hairy)	*Leea guineensis*
Bagged	24	22	12
Unbagged	1	3	0

Table 2. Nested ANOVA with fruit counts for the individual *Leea* plants tested and for bagged and unbagged treatments as input variables.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Individual *Leea* plants	0.1547	12.0000	0.0129	1.3822	0.2919
Treatment	0.2025	1.0000	0.2025	21.7213	0.0006
Error	0.1119	12.0000	0.0093		

Visitation. A comparison of visitation frequencies using pooled data for the three *Leea* morphospecies shows that there is visitor discrimination among flowering stages, with stage 4 having the most visits and stage 6 with the least visitation (Fig. 2A). The difference is significant for *L. guineensis* ($P < 0.003$, one-way ANOVA), but not for the hairy *L. indica* ($P < 0.07$) nor for the glabrous *L. indica* ($P < 0.11$). There was also significant difference among visitation frequencies for each insect (Fig. 2B; two-way ANOVA, $df = 11$, $F = 2.8396$ $P < 0.0179$), but not between *Leea* morphospecies ($df = 2$, $F = 2.1060$, $P < 0.1456$). Figure 2B compares the percentage of visitation frequencies (N=3,926) of insects common to at least two *Leea* morphospecies.

Leea guineensis received more than twice the number of visitations for either

forms of *L. indica*. *Leea guineensis* attracted 12 species of visitors including four wasp species (Fig. 3H, I, M, one not shown), five butterfly species (Q, T, three not shown), two bee species (J, L) and one fly species (F). Two *L. guineensis* individuals growing in an open area about 2 km from the plot were frequented by additional visitors (C, D, G, K, N, O, P, R, S) including two kinds of spiders (A, B) that are unlikely pollinators. The hairy *L. indica* attracted seven visitor types (D-F, I, J, L, Q), while the glabrous form was seen frequented by the same kinds that visited the hairy form excluding *Ragadia luzonica* (Q) and the unidentified weevils (D, E). It was also visited by plume moths (U). The spider wasp (I) was observed in all three *Leea* species, comprising 50 percent of visitations for the glabrous *L. indica*, and only 9 and 34 percent for the hairy *L. indica* and *L. guineensis,* respectively. The bee, *Nomia* sp. (L), was also a constant visitor, making up 40, 4, and 10 percent of visitations for the hairy *L. indica*, glabrous *L. indica,* and *L. guineensis*, respectively.

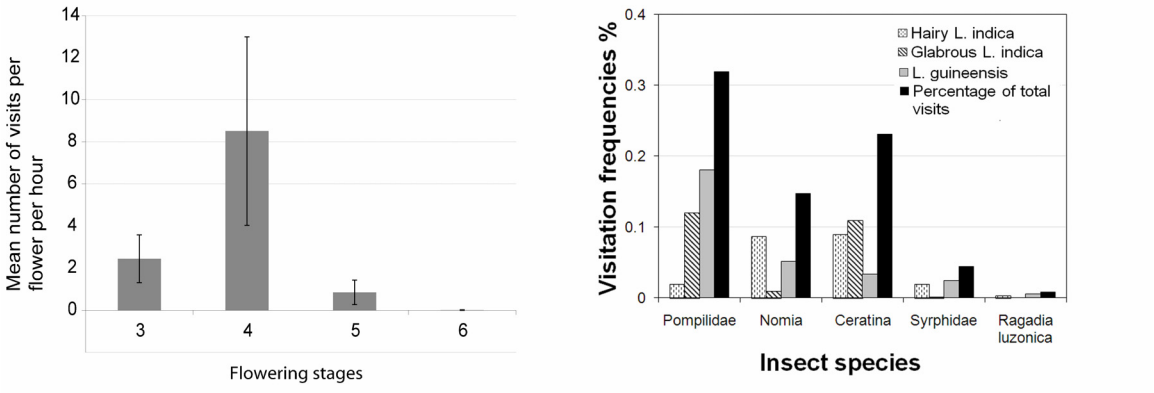

Fig. 2. A. Mean visitation frequencies of the different stages using pooled data for the three morphospecies; B. Percentage of visitation frequencies (N=3,926) of insects common to at least two *Leea* morphospecies. Taxa that visited *Leea guineensis* but not either *L. indica* forms are not shown.

DISCUSSION

Flower development. Flowers undergo morphological changes that signal commencement and termination of male and female phases. The disclosure of the gynoecium after abscission of the stamens marks the beginning of the female phase, as shown by the positive bubbling reaction of the stigma upon contact with hydrogen peroxide. Style length of female-phase flowers was also 37.5 percent longer than that of male-phase flowers showing that style length grows considerably as the stigma becomes receptive to pollination (Devlin and Stephenson 1985).

Though late stage 4 flowers tested to have receptive stigmas, the stamens' attachment on top of the floral tube effectively shields the stigma from open pollination, which makes this stage essentially male-phase. Gerrath et al. (1990) observed that *Leea* flowers in cultivation did not complete development past the male phase. I have also observed that stigmas of the ornamental species *Leea coccinea* Planch. grown in a greenhouse tested negative with hydrogen peroxide even after natural abscission of the stamens. This may be explained by the absence of pollen gathering by insects in the greenhouse which may be necessary to transition into the female phase. The onset of the female function is said to be affected by the rate at which pollen is harvested (Bhardwaj and Eckert 2001).

Synchronized dichogamy. The progression from a distinct male phase to female phase demonstrates an effective strategy to avoid autogamous pollination and its potential costs. However, this does not safeguard against geitonogamy. Of the three *Leea* morphospecies, only *Leea guineensis* exhibited some synchronized dichogamy, which

may be most important in this species as it has more flowers in its inflorescence, which could predispose it to a greater degree of geitonogamy (Galloway et al. 2002). Forty-seven percent of the open flowers of the hairy *L. indica* and 36% of those of the glabrous *L. indica* were unisexual at one time. These were not significantly greater from the simulated PNO's, which may be due to a natural tendency towards geitonogamous selfing, especially for the glabrous *L. indica,* which is clonal, or to inadequate sampling. Further testing is necessary to confirm this. It is possible that the white-flowered *L. indica* morphs resort to frequent selfing, perhaps due to lesser visitation in contrast to the red-flowered *L. guineensis* (see results for insect visitation). Epperson and Clegg (1987) observed a similar pattern in which white flowers of *Ipomoea purpurea* were under-visited and had lower outcrossing rates compared to colored flowers.

Autonomous autogamy. Bagged treatments produced significantly less fruit. It is possible that this may have even been facilitated by ants, which were seen on the inflorescence of one of the glabrous *L. indica* individuals sampled. Control treatments had 12, 22, 24 percent fruit production for *L. guineensis,* hairy *L. indica,* and the glabrous *L. indica* (Table 1), respectively, which is comparable to fruit set in grapes (Mullins et al. 1992) and in the grape relative *Cayratia japonica* (Kakutani et al.1989), where less than 30 percent of the total bud count become fruits. Significantly diminished fruit set in bagged treatments demonstrates *Leea's* inability to be autogamous and its need for insect-mediated pollination, though it is unclear whether it requires insect-mediated selfing, outcrossing, or both. Previous flower emasculations by hand to determine *Leea's* potential reliance on insect-mediated outcrossing were futile, resulting in aborted flowers the following day (J. Molina, pers. obs.), perhaps due to inadvertent mechanical injury or

lack of pollen-gatherers that may be necessary to progress to female phase.

Environmental correlates. Flowers of the hairy *Leea indica* were positively affected by light. This was similar to the response found in grapevines (*Vitis* spp.), which showed an increase in the number and size of inflorescence primordia with increases in light intensity (Buttrose 1968). The buds and open flowers of the hairy *L. indica* did not show a significant positive association with ambient temperature. However, the average temperature recorded for the three hairy *L. indica* individuals throughout its phenology was 29.08±0.21 ^{o}C, which is very close to the favored thermal conditions of grapes. Grapevines require high temperatures (*ca* 30 ^{o}C) to induce the maximum number of inflorescence primordia (Buttrose 1969). The number and size of the inflorescence primordia in grapes is also reduced by water stress (Buttrose 1974). Though there was no significant relationship between *Leea* bud or floral counts and soil moisture, the average moisture content for the hairy *L. indica* was 8.77±0.20 out of a maximum 10 on the Sunleaves® moisture scale, indicating that it favors wet conditions, like grapevines. All three morphospecies were also found on the edge of stream banks. The average soil pH of 7.15±0.05 for the hairy *L. indica* was also close to the pH range recorded for three *Vitis* species in the wild (Morano and Walker 1995).

Insect visitation. *Leea* is visited by a diversity of insects, in addition to the halictid bees and syrphid flies, reported previously (Ridsdale 1976). All three *Leea* morphospecies were also seen frequented by ants, which are possibly attracted to their stem extrafloral nectaries (Fiala and Linsenmair 1995). Greater visitation of winged insects on male-phase flowers (Fig. 2) may be explained by an emphasis on male function at anthesis, which may promote greater pollen dissemination in hermaphrodites

(Devlin and Stephenson 1985). The preference for male-phase flowers may also reduce geitonogamous pollen transfer (Bhardwaj and Eckert 2001, Galloway et al. 2002). This is consistent with Bell's (1985) hypothesis that male flowers receive increased visitation than female flowers due to greater resource allocation (e.g. larger and more attractive male flowers in sexually dimorphic plants). Though the presence of nectar was not tested in this study, I have observed that male flowers produce a clear exudate, that was more pronounced in bagged setups which are inaccessible to insects. The potential presence of nectar is contrary to published literature (Gerrath et al. 1990).

The red-flowered *L. guineensis* attracted more visitations from more visitor types than either morphospecies of *L. indica.* This bias may be attributed to flower color, wherein different insects may discriminate against white UV-reflecting flowers (Chittka 1999) in preference for UV-absorbing pigmented phenotypes due to their possession of receptors for the latter (Briscoe and Chittka 2001).

The two morphospecies of *L. indica* have synchronous flowering phenologies. It is possible that these two hybridize because they share the same pollinator assemblage. The existence of a third white-flowered form of *L. indica* (not studied), that is intermediate in height and pubescence between the two studied morphospecies (J. Molina, pers. obs.), within the vicinity of the plot, may be evidence of this possible hybridization.

This is the first field study of *Leea's* reproductive biology. The short life span of its flowers and their readily observable sexual phases make *Leea* a good system for studying the biology of protandrous bisexual flowers. The existence of mechanisms for protandry and synchronized dichogamy, as well as its inability to self autonomously

suggest that *Leea,* particularly the red-flowered *L. guineensis,* may have evolved floral traits to avoid selfing and its costs. This may not be true for the white-flowered morphospecies, which may be more predisposed to geitonogamous selfing. Though *Leea* is similar in floral morphology with its grape relatives, its unique floral tube sets it apart from the Vitaceae. Given the observed overlap in male and female maturity in stage 4 flowers, the *Leea* floral tube may be necessary in preventing pollen-stigma interference, which may be inevitable in the absence of this structure. The grape relatives, *Ampelopsis brevipedunculata* (Gerrath and Posluszny 1989)**,** *Cayratia japonica* (Kakutani et al. 1989)*, Cyphostemma* (Wilson et al. 2006)**,** *Parthenocissus* (Wilson and Posluszny 2003)**,** *and Rhoicissus digitata* (Gerrath et al. 2004) all lack a floral tube, like all other Vitaceae (Wen 2007a, b), and this may theoretically predispose them to self-pollination since the stamens and stigma simultaneously exist in the flower. Developmental studies (as cited above), however, have shown that they all undergo progressive morphological changes that exhibit a protandrous mechanism, in spite of the lack of the tube. More comparative studies are needed to fully understand the evolutionary significance of *Leea's* specialized floral tube.

NOTE TO THE READER

This manuscript has been submitted to *Plant Species Biology* (Blackwell Publishing) in June 2008 and has been accepted for publication in July 2008. This is the revised and most current form of the article submitted to the journal as of November 2008, and has remained unaltered in this dissertation. Thus, taxon names used herein do not reflect the tentative species names recently proposed in chapter 2 of this dissertation.

For the sake of nomenclatural uniformity across all four chapters, the white-flowered morphospecies (*Leea indica* sensu Ridsdale) examined for floral biology studies include *L. cumingii* Clarke (pubescent, small tree) and *L. palanensis* sp. nov. J. E. Molina (glabrous sub-shrub). The red-flowered morphospecies (*L. guineensis* sensu Ridsdale) refers to *L. luzoniensis* Elmer.

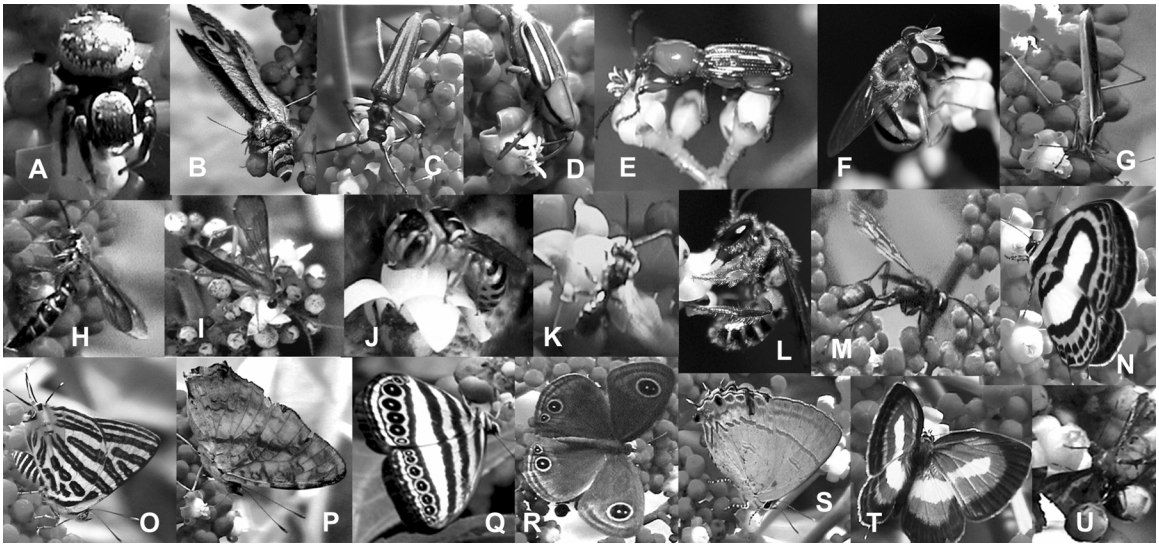

Fig. 3. Visitors of *Leea*. (A-B) Araneae. Species unidentified (both Salticidae, B with butterfly prey); (C-E) Coleoptera (C) *Chloridolum accensum* (Cerambycidae), (D-E) Weevils, species unidentified (Curculionidae); (F) Diptera, species unidentified (Syrphidae); (G) Hemiptera, *Leptocorisa* sp. (Coreidae); (H-M) Hymenoptera (H) *Campsomeris* sp. (Scoliidae), (I) Spider wasp (Pompilidae), (J) *Ceratina* sp. (Apidae), (K) *Braunsapis philippinensis* (Apidae), (L) *Nomia* sp. (Halictidae), (M) *Sphex* sp. (Sphecidae); (N-U) Lepidoptera (N) *Discolampa ethion* (Satyridae), (O) *Spindasis syama* (Lycaenidae), (P) *Symbrenthia lilaea* (Nymphalidae), (Q) *Ragadia luzonica* (Satyridae), (R) *Ypthima sempera* (Satyridae), (S-T) Species unidentified (both Lycaenidae), (U) Plume moths (Pterophoridae). Photos E, F, L, courtesy of Sandra Yap.

CHAPTER 4: Biogeography of the grape order Vitales with focus on the non-viny Leeaceae: Laurasian Tertiary dispersals to Gondwanan continents

ABSTRACT

The monotypic Leeaceae is sister to the grape family, Vitaceae, and both make up the order Vitales, which is regarded as one of the more early-diverging angiosperm groups. Previous studies have elucidated the infra-familial relationships within Vitales, but detailed biogeographical hypotheses to explain the current distribution of this diverse group are lacking. To understand the paleotropical distribution of the non-viny Leeaceae, divergence times calibrated with fossil dates and putative ancestral areas were inferred using the estimated molecular phylogeny of the family. Biogeographic scenarios were also presented for its order Vitales, whose fossils abound in the early Tertiary of Laurasia, in an attempt to locate the ancestral origin of the *Leea* stem lineage. It is likely that the parent lineage of *Leea* originated in Europe and dispersed into late Cretaceous India and/or Indochina, where the crown group evolved. Both were in close proximity during this time, which may have allowed biotic exchange of vagile taxa, since India was drifting north, eventually colliding with Asia in the early Tertiary. The dynamic geological and environmental changes occurring in Neogene Southeast Asia stimulated rapid diversifications in *Leea* resulting in the formation of many cryptic species with wide geographical distributions. Asian species reached Australia in the late Oligocene prior to the emergence of potential stepping stone islands, but this needs to be confirmed by adequate taxon sampling. Species in Africa and Madagascar also arose from Asian

ancestors, which dispersed in mid-Miocene, an epoch marked by warm, wet, and equable climes that permitted rainforests to expand. Divergence between Indochinese and West Malesian taxa occurred subsequent to the Miocene flooding of the Thai-Malay border. *Leea* speciations were promoted by Neogene (23-1.8 mya) changes and not by Pleistocene glaciations (1.8 mya-10000 yrs before present).

INTRODUCTION

Leea systematics is rife with species complexes (Molina, chapters 1, 2). The current phylogeny of *Leea* using nuclear markers confirmed the polyphyly of *L. indica* and *L. guineensis,* as well as the geographic cohesion of many entities (Molina, chapter 1). It also revealed that *L. asiatica,* a species ranging from India to mainland Southeast Asia, may be composed of two morphotypes (*L. asiatica* s. str. and *L. aspera*) corresponding to previously described species that have been synonymized (Ridsdale 1974). The phylogeny also presented four robustly supported major clades. *Leea asiatica* s.l. (Clade I) is sister to the other three clades, and has retained the plesiomorphic character of free stamens, a feature unseen in other species of *Leea,* but displayed by members of its sister family, Vitaceae. Spine-bearing *Leea* species (Clade II) were grouped as sister in the parsimony analysis to *Leea* species possessing distinctively larger flowers and leaves endemic to the primary forests of Borneo, New Guinea, and the Philippines (Clade III). Clades II+III were recovered as sister to Clade IV, comprised of the notoriously difficult species complex *L. indica* and *L. guineensis,* as well as other

morphologically distinct species (*L. aequata, L. compactiflora, L. longifoliola, L. macrophylla, L. rubra, L. setuligera*).

Fig. 1. Map of Southeast Asia including Indochina (Myanmar, Thailand, Cambodia, Laos, and Vietnam) and Malesia (Malaysia, Indonesia, Brunei, Singapore, Philippines, and Papua New Guinea). Indomalaya refers to the collective region of India, Indochina and Malesia.

Like many paleotropically distributed groups, *Leea* is highly diverse in the Malesian region, a distinct floristic zone, composed of Malaysia, Indonesia, Brunei, Singapore, Philippines, and Papua New Guinea (Fig. 1). Malesia is bordered in the northwest by Vietnam, Cambodia, Laos, Thailand, and Myanmar, which collectively comprise mainland Southeast Asia or Indochina. It is believed that many taxa in Malesia were derived from biotic elements that arrived in Asia through the Indian plate when it rafted away from the rest of Gondwana (Africa, South America, Antarctica, Madagascar, Australia) beginning in early Cretaceous (120 mya). This out-of-India hypothesis

(McKenna 1973) has been supported for Crypteroniaceae (Conti et al. 2001; Rutschmann et al. 2004) and Dipterocarpaceae (Dayanandan et al. 1999), whose members are now only confined in Southeast Asia. Southeast Asian Melastomataceae also exemplify this biogeographic pattern (Morley and Dick 2003). The close allies of these families occupy basal positions (i.e. early-diverging) in current phylogenies and are found in South America and Africa implicating Gondwanan vicariance. Fossils with affinities to Dipterocarpaceae and Melastomataceae have also been exhumed in Tertiary India, Madagascar and Cretaceous West Gondwana (Africa+South America, Morley 2000; Morley and Dick 2003), attesting to India's role as a "biotic ferry" for Gondwanan taxa. Their extinction on the Indian plate may have been precipitated by Neogene aridification (Morley 2000; Conti et al. 2001)

The out-of-India hypothesis has also been corroborated by phylogenetic and paleontological evidence from several faunal groups such as ranid frogs (Bossuyt and Milikonvitch 2001), acrodont lizards (Macey et al. 2000), caecilians (Gower et al 2002), ammonites (Bardhan et al. 2002), arowanas (Kumazawa and Nishida 2000), and whales (Thewissen et al. 2007). Krause and Maas (1990) argued that India may have been the center of origin for many evolutionary lineages. Sahni (2006 and citations within) reviewed the oldest fossil records found for some vertebrate groups, including perciform fishes, Asian catfishes and Colubrid snakes, and ostracodes. The fossils of the gobiid fishes emerged in the middle Eocene of Europe, USA, and Java, but the oldest record had been found in the lower Eocene of India (Bajpai and Kapur 2004). Gower et al (2002) also elucidated the phylogeny of the ichthyopiid caecilian family and showed that Indian lineages were more "basal" and older than the southeast Asian members.

Nonetheless, there are also taxa that show the opposite trend, which Hedges et al. (1993) coined as the "out-of-Asia" hypothesis. Koehler and Glaubrecht (2007) have demonstrated this pattern using an Indian-endemic freshwater gastropod, which the phylogeny showed as nested within Southeast Asian taxa. They believed that the out-of-Asia migration took place during the Eocene collision of the Indian and Asian plates. An oriental origin was also demonstrated for tiger frogs (Kosuch et al. 2001) as well as in the paleotropical herb *Impatiens* (Balsaminaceae), which seems to have originated in South China and then colonized the adjacent regions of India and Southeast Asia (Janssens et al 2006).

Support for either of these two hypotheses--out-of-India or out-of-Asia--is anticipated by recalibrating the current molecular phylogeny of *Leea* with fossils and known geological events and superimposing these age estimates on reconstructed ancestral states. The oldest fossil resembling *Leea* was discovered in central India, specifically between the Deccan Traps of Nagpur and is taxonomically identified as *Leeoxylon* (Prakash and Dayal 1964). The Deccan Traps represent a large igneous province coated by several layers of flood basalt derived from episodic volcanic eruptions at around 65 mya (Allègre et al. 1999). This date will be used to calibrate the root of the phylogeny, while one Miocene fossil from Japan, *Leea eojaponica* (Watari 1951), will provide a second calibration point for an internal node. Two other fossils, though with *Leea* affinities, will not be used for calibration since they cannot be matched to any living species. These are *Leeoxylon altiradiatum* from the Neogene of Java (Kramer 1974) and *Carpolithus olssoni* (Berry 1929; Chen and Manchester 2007) from the Eocene of Peru.

To provide a broader perspective on the origin and biogeography of *Leea*, this paper will also attempt to elucidate the biogeographical history of the order Vitales (Leeaceae+Vitaceae) by estimating divergence dates and ancestral areas on a concatenated matrix of previously published sequences for Vitaceae (Soejima and Wen 2006; Rossetto et al. 2007; Wen et al. 2007). A Laurasian origin was predicted by Chen and Manchester (2007) based on abundant Vitaceae fossil evidence from the Tertiary of North America and Europe. This is tested in this paper and results are discussed in relation to *Leea*'s own biogeography.

MATERIALS AND METHODS

Phylogeny and divergence time estimation

Vitales. In an attempt to reconstruct a comprehensive phylogeny of the order, sequences were downloaded from Genbank for the chloroplast gene *GAI1* (Wen et al. 2007) and *trnLF* spacer (Soejima and Wen 2006; Rossetto et al. 2007), which when concatenated, provided overlapping alignable regions for 14 out of 15 genera (Table 1). The concatenated sequences were aligned using the algorithm *L-insi* in MAFFT (Katoh et al. 2002) producing a matrix of 2760 bases including indels. Each partition was run in MrModeltest separately to derive the best fitting model for DNA substitution. MrModeltest (Nylander 2004) recommended GTR+G for each partition under the Aikake criterion.

The phylogeny and divergence time of splits were estimated in BEAST v1.4.8 (Drummond and Rambaut 2007). BEAUTI, a program that comes with the software package, was used to set the model parameters for the BEAST analysis. The uncorrelated relaxed molecular clock model (Drummond et al. 2006) was implemented using the default uniform priors on the mean, standard deviation, GTR substitution parameters, and gamma shape parameter. I have employed the Yule Tree prior, which assumes a constant speciation rate per lineage, as suggested in the BEAST manual (Drummond et al. 2007) for species-level phylogenies.

Certain taxa were constrained to be monophyletic (all *Leea*, all *Tetrastigma*, *Clematicissus+(Cissus striata+Cissus tweediana)*, all *Rhoicissus*, all *Vitis*) based on previous phylogenetic analyses (Soejima and Wen 2006; Rossetto et al. 2007; Wen et al. 2007). The nodes were calibrated using the following dates as minimum ages: 1) 65.6±0.3 mya for the *Leea* clade. This is the time of the Deccan Trap eruptions (Allègre et al. 1999), which yielded *Leeoxylon* fossils between the basaltic beds; 2) 48.6 mya for each of the *Tetrastigma, Rhoicissus,* and *Vitis* clades. This date refers to the lower boundary of the early Eocene epoch, when the oldest fossils of the above Vitaceae genera were collected (Manchester 1994; Morley 2000). A uniform distribution (108-117 mya) was specified for the root height prior corresponding to previous age estimates for the order (Wikström et al. 2001; Anderson et al. 2005). Two independent MCMC runs (auto-optimize option selected), each with 25000000 iterations, were executed (samplefreq=500) and combined in LogCombiner v.1.4.8 (included in the BEAST package) to estimate the model parameters and divergence dates. The log output files were also analyzed in Tracer v1.4.8 (included in the package), which suggested that

ample number of iterations were conducted as depicted by large effective sample sizes (ESS, >100). A consensus tree was produced in TreeAnnotator v.1.4.8 by discarding 25% of the samples.

Leeaceae. A smaller data matrix from chapter 1 (Table 2) was used as the input file in BEAST to estimate divergence times. The Aikaike criterion in MrModeltest recommended GTR+G+I for the concatenated ITS and 5S-NTS dataset. A preliminary BEAST analysis of the dataset employing the uncorrelated lognormal relaxed clock yielded a standard deviation < 1, which is indicative of the data being quite clock-like (Drummond et al. 2007). Thus, a strict molecular clock was subsequently enforced using the best-fitting model and default parameters.

Some taxa were assumed to be monophyletic based on previous phylogenetic findings (Molina, chapter 1), and their clades were calibrated with fossils or known geological evidence. The ingroup was constrained to be monophyletic with its root assigned a normal distribution with mean of 65.6±0.3 mya, which is the estimated age for the Deccan Traps (Allègre et al. 1999), where *Leeoxylon multiseriatum* was collected. The *L. angulata-L. spinea* clade was specified a uniform distribution (5-23 mya) corresponding to the upper and lower bounds of the Miocene epoch, which is the estimated age for *L. eojaponica.* This fossil closely resembled the wood of *L. angulata* (Watari 1951). Since land only became available in Taiwan by the Pliocene, the Taiwan-North Philippine sister relationship could have developed during this time, which was used to calibrate the node shared by *L. guineensis WA6727* from Taiwan and *L. guineensis MO18* from Luzon, Philippines. The root height prior (Leeaceae-Vitaceae split) was also constrained as in the Vitales analysis with a uniform distribution ranging

from 108-112 mya. Two independent MCMC runs of 5000000 steps each were sufficient to achieve ESS values>> 200. These were combined in LogAnnotator to approximate the dates of the nodes. Twenty-five percent of the samples were discarded in TreeAnnotator to produce a consensus tree.

Ancestral Area reconstructions

Vitales. Ancestral areas were inferred in DIVA v1.2 (Ronquist 1997), which uses a parsimony-based method. It assumes that speciation are a result of vicariance, thus vicariant events are not counted as steps, but changes in distribution interpreted as extinctions or dispersal are assigned a cost. For the Vitales analysis, a matrix of areas corresponding to extant and known fossil distributions (fossil: Muller 1981; Morley 2000; Poole and Wilkinson 2000; Chen and Manchester 2007; extant: Soejima and Wen 2006; efloras.org) was created. Areas (Fig. 2) included A) South America; B) North America; C) Europe; D) India; E) Australia; F) Southeast Asia; G) Africa; and H) Northeast Asia. Default settings were employed with the maximum number of areas (maxareas) unconstrained to allow for multiple dispersals. Given the widespread occurrence of its fossils, the Vitales ancestor is hypothesized to have been dispersed easily. Areas were optimized on the BEAST topology, which was slightly modified (Fig. 2) to reflect the *GAI1* phylogenetic relationships for *Ampelopsis*-*Rhoicissus*-*Clematicussus*-*Cissus striata/tweediana* published by Wen et al. (2007) since the original BEAST topology from this analysis showed weakly supported relationships for these taxa.

Leeaceae. *Leea* ancestral areas were inferred by coding species as belonging to any of these twelve areas (Fig. 3): A) tropical Africa; B) Madagascar and adjacent islands (Mauritius, Comoros); C) India; D) Indochina; E) West Malesia (Malay Peninsula, Sumatra, Java, Borneo, Palawan); F) New Guinea including the Bismarck Archipelago; G) Northeast Australia; H) Fiji; I) Solomon Islands; J) Philippines; K) Taiwan; L) Palau. Areas were optimized on the BEAST topology using DIVA (Fig. 3). Since the Miocene Japanese fossil *L. eojaponica* (Watari 1951) was morphologically similar to the modern species of *L. angulata,* Japan (M) was also added to its list of distribution areas. The maximum number of areas was set to 3 to represent the geologically ancient areas of India, Indochina, West Malesia (Sumatra, North Borneo) that were in close proximity (Burrett et al. 1991) when *Leea* was believed to have originated sometime in the late Cretaceous (c. 65 mya), as evidenced by the oldest fossil recovered from India.

RESULTS

Vitales. The split between Vitaceae and Leeaceae was estimated at 112.41 mya. Based on the available data the Vitaceae ancestor was widespread during the mid-Cretaceous (Fig. 2). The two major clades I and II within Vitaceae diverged 108.37 mya (age of Vitaceae). The ancestral area of clade I is placed in India (D), which used to be part of Gondwana, whereas clade II originated in Laurasia (BCH). Both clades I and II evolved in the early late Cretaceous (93.5-99.6 mya).

Within clade I, the ancestor of the *Cissus* s. str. clade (I-1, excluding *C. striata/C. tweediana* from South America and Australian *Cissus*) was present in both India (D) and

Africa (G) in the Paleocene (64.6 mya), while the ancestor of *Cayratia/Tetrastigma/ Cyphostemma* (clade I-2) evolved earlier in middle late Cretaceous (84.7 mya) in India. Subclade II-1 originated in Europe (C) in the late Cretaceous (75.9-78.5 mya). The *Yua/*Australian *Cissus/Parthenocissus* (subclade II-2) evolved in the Eocene (50.3 mya) in Northeast Asia (H). North America (B) was reconstructed as the source of subclade II-3. Since species sampling was limited, generic ages may be unreliable and were omitted (except for the fossil calibrations, Fig. 2) and only major splits were discussed.

Leeaceae. The root of *Leea* was calibrated at 65.6±0.3 mya. Three major clades (I, II, III) started to diversify in the Miocene (23 mya) except for clade IV, which split very late in the Oligocene (25.6 mya; Fig. 3). Clade IV has 4 subclades (a-d) all diversifying in the Neogene (Miocene-Pliocene, 23-1.8 mya). Subclade "d" is broken up further into clades d1, d2, d3, and d4.

Area optimization placed the *Leea* ancestor in the combined regions of India and Indochina (CD). Clade I originated in India and Indochina (CD). The ancestor of both clades II+III was in West Malesia (E) during the late Eocene (36.3 mya), while that of clade IV was present in both Indochina and Australia (DG) in the late Oligocene (25.6 mya) suggesting an ancestral distribution encompassing Indochina, West Malesia, and Australia (DEG) in early Eocene (50.8 mya).

Clade II's ancestor dispersed from the ancestral source of West Malesia (E) to Madagascar/Mauritius/Comoros (B), Philippines (J), and Japan (M). The entire Malesian region (EF, i.e. West Malesia plus New Guinea) was reconstructed as the birthplace of clade III, with the islands of Philippines (J) and New Guinea (F) harboring the more derived endemic species. Subclade "a" of clade IV probably originated in Australia (G)

followed by dispersals to the Pacific Islands of Fiji (H) and Solomon (I), with the youngest members in New Guinea (F). Subclades b, c, and d each evolved in Indochina (D). Subsequent dispersals to tropical Africa (A), Madagascar/ Mauritius/Comoros (B) (see subclade "c"); West Malesia (subclade d2); and Philippines (subclade d4) took place in late Miocene. Both *L. aequata* and *L. rubra* were widespread throughout Indomalaya (CDEF) in the Pliocene, while Taiwan (K) and Palau (L) were also colonized by Philippine *Leea* during this time.

DISCUSSION

Biogeography of Vitaceae

Bayesian divergence time estimation yielded a topology that is almost congruent with phylogenies previously published for Vitaceae (Soejima and Wen 2006; Wen et al. 2007), except that the pantropical *Cissus* s. str. clade (I-1, Fig. 2; clade 2 sensu Soejima and Wen 2006) is recovered as sister to the paleotropical *Cayratia-Tetrastigma-Cyphostemma* clade (I-2; clade 3 sensu Soejima and Wen 2006). This tropical clade is then sister to a clade including the rest of the genera in the family (II in Fig. 2; clade 1 sensu Soejima and Wen 2006).

Soejima and Wen (2006) and Wen (2008) reiterated that complex biogeographical patterns underlie Vitaceae's phylogenetic history. Chen and Manchester (2007) based on fossil evidence postulated a Laurasian origin for the family, but ancestral area analysis inferred a widespread ancestor present in both Laurasia and Gondwana in mid-Cretaceous (112 mya). At this time the northern landmass Laurasia was already separate

from the southern Gondwana by the Tethys Ocean, with Gondwana already broken up into western (South America, Africa) and eastern (Madagascar, India, Seychelles, Antarctica, Australia) regions (Ali and Aitchison 2008). Laurasia was already divided into the Nearctic (North America) and Palearctic (Europe and Asia or Eurasia) rifted by the newly formed shallow Atlantic sea (Cox and Moore 2005).

Palynological records document the rise of the angiosperms in both Laurasia and Gondwana in the late Cretaceous perhaps encouraged by the warmer (20-25^{o}C) subhumid climes that persisted in these hemispheres even at high latitudes (>60^{o}N, S; Morley 2000, 2003). It is presumed that vegetation started as small herbs and shrubs (open canopy) with a few vines (Wing and Boucher 1998). It is not hard to imagine that some of these vines may have been the precursors of extant Vitaceae, which evolved perhaps towards the end of late Cretaceous (108 mya, Fig. 2; Wikström et al. 2001; Anderson et al. 2005).

Divergence between the two major clades of Vitaceae (I, II) occurred in the earlier late Cretaceous (93.5-99.6 mya, Fig. 2), with clade I originating in India, when it was still juxtaposed with Madagascar and both lay close to other Gondwanan continents (Africa, Australia). This proximity perhaps permitted interplate dispersals of members in clade I, as such crossings have been documented for other megathermal plants (Morley 2003), thus *Cissus* s. str., *Cayratia, Tetrastigma,* and *Cyphostemma* are presently found in these southern continents. Portions of the Kerguelan plateau that were subaerially exposed in between Australia and India until the beginning of the late Cretaceous (83.5 mya) may have also served as a passageway for crossing biota (Ali and Aitchison 2008).

As India drifted northward and became more appreciably isolated from the southern continents by 67.7 mya (Ali and Aitchison 2008), *Cayratia* and *Tetrstigma* may

have begun their migration into Laurasia. *Tetrastigma* fossil seed was collected in early Eocene Europe (London Clay), along with seeds of other Vitaceae genera such as *Ampelopsis, Parthenocissus,* and *Vitis* (Manchester 1994; Morley 2000). This ancient distribution coincided with the Paleocene-Early Eocene global thermal maximum, which witnessed the zenith of rainforest diversification due to the warmer equable climates that persisted even at higher latitudes. On the other hand, *Cyphostemma,* being presently most diverse in Africa and Madagascar, is likely to have had its stem group dispersed into Africa from India. Though taxon sampling was limited in this study, the age of *Cyphostemma* falls within the Oligocene, when Africa was experiencing drier climates (Morley 2000), hence it is not surprising that a pachycaul habit was selected for as an adaptation to this arid condition.

Laurasia, comprised of North America, Europe, and Northeast Asia in this analysis, was inferred to be the ancestral source of Clade II (Fig. 2). Clade II-1 (*Ampelopsis, Rhoicissus, Clematicissus, Cissus striata-C. tweediana*) originated from Europe in the late Cretaceous (78.5 mya), which was when frost-free humid climates prevailed across the mid-latitudes (Morley 2003). South Greenland (45-50^{o}N) afforded a passageway for megathermal taxa between Europe and North America (Morley 2003), which would explain the occurrence of modern *Ampelopsis* here. Though *Ampelopsis* species are also found in Asia, it may have not been possible for the European ancestral population to have dispersed eastward due to the wide Turgai seas separating Europe and Asia until the Oligocene. Thus, it is more likely that American *Ampelopsis* migrated through the Bering land Bridge that connected North America with Asia (Tiffney and Manchester 2001). This land connection was too far north (75^{o}N) and would have only

permitted crossings of temperate taxa such as *Ampelopsis.* Meanwhile, *Rhoicissus*, which is presently endemic to Africa, was inferred to have originated in Europe, and this is supported by fossil wood strongly resembling *Rhoicissus* that was excavated from the early Eocene of southeast England (Poole and Wilkinson 2000). The onset of global cooling in mid-Eocene may have compelled tropical *Rhoicissus* to seek refuge in warmer Africa, which would explain its absence in present-day Europe.

Since the interrelationships in Clade II-1 are weakly supported in the original BEAST topology, the branching patterns within this lineage was slightly modified for ancestral area optimization. In Wen et al.'s (2007) analysis, *Rhoicissus* was recovered as sister to *Cissus striata.* However, they did not include *Cissus tweediana* and *Clematicissus*, which have been shown to be closely associated with *Cissus striata* by Rossetto et al. (2007), such that the Australian *Clematicissus* is sister to the South American *Cissus striata+C. tweediana* (Chile, Argentina, respectively). Integration of these relationships together resulted in *Rhoicissus* being sister to *Clematicissus+(Cissus striata+C. tweediana)*, which poses an interesting biogeographical scenario wherein their common ancestor presumably traversed the then-warmer Antarctic coast during the global thermal maximum, with subsequent vicariance due to late Eocene cooling. This was the same dispersal route taken by originally South American taxa (Bombacaceae, Cunonianiaceae, Didymelaceae, Myrtaceae, etc.) that are now found in Australia (Morley 2000, 2003).

Northeast Asia was hypothesized as the birthplace of clade II-2 (*Parthenocissus, Yua,* and the Australian *Cissus*). Modern *Parthenocissus,* like *Ampelopsis,* shows a disjunct distribution between Asia and North America, which may also be explained by

migration of this temperate taxon across the Bering land bridge. Meanwhile, the close relationship between the Australian endemic *Cissus* and the predominantly Eastern Asian *Parthenocissus* is an interesting alliance purporting a southward dispersal of Asian elements into Australia prior to the emergence of potential stepping stone oceanic islands in Southeast Asia. This dispersal pattern was also supported by phylogenetic evidence from the fern *Polystichum*, which potentially migrated from China into Australia in the Eocene (Li et al. 2007), an age also reflected in the *Yua*/*Parthenocissus*/Australian *Cissus* split resulting from the BEAST analysis (Fig. 2).

Clade II-3 (*Ampelocissus, Vitis, Nothocisuss, Pterisanthes*) was predicted to have originated from North America, which harbored abundant fossils of both *Ampelocissus* and *Vitis,* including the oldest fossil seed of the former collected from the Paleocene of North Dakota (Chen and Manchester 2007). Current *Vitis* distribution also portrays the same North American-Northeast Asian disjunction for temperate flora, presumably arising from an eastward dispersal of North American *Vitis* across the Bering land bridge. The strictly Malesian genera of *Nothocissus* and *Pterisanthes*, which Chen and Manchester (2007) considered as indistinguishable from *Ampelocissus* based on fossil seeds, are independently nested within predominantly North American clades possibly suggesting southward migration of these taxa into the Malesian tropics as Laurasia's climate became intolerable to megathermal taxa. This southward dispersal pattern has also been documented by Tiffney (1985) in 34 other boreotropical genera now restricted to the Southeast Asian forests.

It must be noted that the above biogeographical patterns are still conjectural, and extensive taxon and geographical sampling for individual genera along with prudent

dating analyses are necessary to test these hypotheses. Nonetheless, discovery of fossils belonging to several Vitaceae genera in the Paleocene and Eocene suggests that generic diversification was mostly complete at that time. Tracing Vitaceae's history sets the biogeographical theater in which we can start to understand the origin of its sister family, Leeaceae.

Biogeography of Leeaceae

As the Vitales ancestor was predicted to be widespread in mid-Cretaceous, it is difficult to speculate on the origin of *Leea*'s stem lineage. However, area optimization traced the crown group back to India and Indochina, which were in close proximity towards the end of the Cretaceous (65.5 mya; Burrett et al. 1991) when the oldest record of *Leea* was exhumed from the Deccan Traps of India. Assuming that *Leea's* ancestor was also bird and sea-dispersed, which had been reported for its modern species (Wen 2007a), then *Leea* would have been able to surmount the intervening Tethys between India and the Indochina block to create this ancestral distribution. Support for such dispersal hinges on the Laurasian affinity of many fossil types embedded in the late Cretaceous Deccan traps of India (Prasad and Khajuria 1995; Soler-Gijon and Lopez-Martinez 1998). Though this model complicates the dichotomy between Hedges out-of-Asia (i.e. excluding India) and McKenna's out-of-India hypotheses, it offers a testable scenario to explain *Leea*'s contemporary distributions and those of other similarly distributed vagile plant groups (*Rhododendron* section *Vireya,* Brown et al. 2006; *Aglaia,* Muellner et al. 2008).

Given the preponderance of Vitaceae fossils in Europe, it is likely that the *Leea* parent taxon may have originated here and dispersed to India, where the crown group may have evolved, as it was for clade I-1 of Vitales. India's proximity to both Europe and Southeast Asia in the late Cretaceous may have allowed the subcontinent to act as a giant stepping stone and cradle of evolution. Smith et al. (2007) observed an eastward migration pattern in bat fossils, which "suggest(s) new palaeobiogeographic scenarios implicating the relative position of India and connections with Europe during the Early Eocene (Smith et al. 2007: 1003)." Tracking the collection localities of Malvaceae fossil woods also revealed this eastward dispersal pattern perhaps with India serving as an intermediary link. For example, the oldest fossil (late Cretaceous) of *Bombacoxylon* was collected from Texas, USA. A late Cretaceous/Eocene wood of the same genus was then found in Ethiopia then subsequently in the Tertiary of Burma. *Hibiscoxylon*, on the other hand, was recorded from the Cretaceous sediments of Egypt and Ethiopia and in the Deccan traps of India (Hinsley 2005-2008 and citations within).

The out-of-India hypothesis is also corroborated by biogeographical patterns of many plant taxa (*Durio, Ixonanthes, Gonystylus, Sonneratia*, see Morley 2000; *Lagerstroemia,* Liu et al. 2007; *Vetiver,* Huq 2000). Floristic assemblages recovered from Java in the middle Eocene were diverse and included novel pollen types that were found in older deposits of India suggesting an eastward influx of Indian taxa (Morley 2000). It is believed that the Indian flora were more aggressive in their invasions resulting in the extinction of many of the flora constituent of the Paleocene and early Eocene Southeast Asia (Morley 2000, 2003). However, area optimization using extant species distribution

did not present this out-of-India scenario, which could emerge with extensive phylogeographic analyses and taxon sampling (Molina, chapter 1).

A complicating factor to the purported origin in India-Indochina is the reported fossil seed closely resembling *Leea* from the Lower Eocene sediments of Peru (Berry 1929; Chen and Manchester 2007). This could suggest that the *Leea* stem lineage may have been widespread in Gondwana prior to its breakup in the early Cretaceous, but this picture would necessitate the occurrence of *Leea* fossils in the other Gondwanan continents of Africa, Madagascar, and Australia, which so far have not been documented. Another possibility is to assume that the strong westerly flowing currents of the Tethys (Crame and Rosen 2002) may have transported *Leea* from its Indomalayan (the collective region of India, Indochina, and Malesia) origin to northern South America followed by subsequent extinction there. As the precise origin of *Leea's* parent lineage is unclear at this point, the following discussion will focus on the crown group's Tertiary diversifications in Southeast Asia assuming its initial evolution in the India-Indochina region towards the end of the late Cretaceous.

Tertiary diversifications

Paleogene (Paleocene-Oligocene: 65.5-23.0 mya). India at the time of the Deccan trap eruptions was experiencing moist equatorial climatic zones (Bande 1992; Mehrotra 2003), which allowed the evolution and diversification of many new tropical lineages, which may have included *Leea*. Prior to its collision with Asia, India drifted very close to southwest Asia (Sumatra and Myanmar) from 57 mya onwards (Ali and Aitchison 2008), which allowed continued floristic dispersals between the two regions.

As presented above, it is possible that *Leea* was one of the Indian migrants that had dispersed into Southeast Asia and had exploited its array of novel habitats. Other taxa with this biogeographical pattern includes Crypteroniaceae (Conti et al. 2001; Rutschmann et al. 2004), Dipterocarpaceae (Dayanandan et al. 1999), *Sterculia, Grewia, Polyalthia, Gomphandra, Lophopetalum, Syzygium*, and *Sonneratia* (Bande 1992; Morley 2000). These tropical taxa are now largely confined to the Malesian region being annihilated by Neogene aridification in India (Morley 2000), which may also be true for the *Leea* species that were once present in India. Only *Leea* taxa that had adapted to drier habitats (deciduous forests or savannah) persist today in the India/Indochina region.

Divergence estimates for *Leea* depict the second split occurring 50.8 mya, with the ancestor of clade II+III+IV distributed in Indochina and West Malesia (Sumatra, Malay peninsula, Northwest Borneo), which formed a contiguous region during much of the Tertiary. India was finally sutured with the Asian plate at c. 35 mya (Ali and Aitchison 2008), which prompted rapid geological and environmental changes that encouraged continued diversification in Southeast Asia (Morley 2000) and may have been responsible for Clade II and III's split. Later in the cooler and drier Oligocene (25.6 mya), Clade IV evolved from an ancestor residing in both Indochina and Australia, though this disjunction may have been an artifact of inadequate taxon sampling, since no entities representing the intervening regions of Java, Sulawesi, and Irian Jaya were sampled (discussed below).

Neogene (Miocene-Pliocene: 23.0-1.8 mya). The Neogene of Southeast Asia was characterized by complex geological and environmental changes, such as the emergence of many oceanic islands, plate convergence, sea level fluctuations and alternating wet and

dry climates (Hall 1998). Sea levels rose in the early Miocene, fragmenting West Malesia such that east Sumatra was submerged and became separated from Indochina and northwest Borneo to which it was once connected (Hall 1998). By mid-Miocene (16 mya), warm and moist conditions allowed proliferation of mixed warm temperate and paratropical forests as far as Japan (Morley 1998). This allowed *Leea* to expand its distribution, which would account for the fossil wood of *Leea eojaponica* collected in the Miocene deposits of Simane, Japan (Watari 1951). This fossil age was used to calibrate the *L. angulata-L. spinea* node, since the fossil wood closely resembled that of *L. angulata* (Watari 1951). *Leeoxylon altiradiatum* could not be precisely identified with any modern taxa (Kramer 1974) and was not used for dating, but its presence in the Neogene of Java could be a preliminary indication of an eastward migration of *Leeoxylon* originating from the older Deccan plate of India. Prolific diversification of Leeaceae in Southeast Asia occurred mid-Miocene onwards, as evidenced in the date estimates of many of the following terminal lineages (Fig. 3). This radiation concurred with the onset of the progressively wetter climes in Southeast Asia following the beginning of the Himalayan uplift (Woodruff 2003).

Clade I. The areas of India and Indochina were reconstructed as the ancestral area of this clade, which originated during the mid-Miocene upheaval of the Himalayas. Members of this clade possess five free stamens, a plesiomorphy not found in any other *Leea*, but shared with Vitaceae members. This clade is composed of two distinct morphotypes: *L. asiatica* s. str. (=*L. crispa* sensu Lawson) and *L. aspera* sensu Lawson (Molina, chapters 1, 2). *Leea asiatica* s. str. occurs in the far east Himalayas (Sikkim, Khasia) to Indochina and Yunnan, China and is a polyploid (2n=48), whereas *L. aspera* is

confined in Western Tropical Himalaya (Jamu to Nepal) all the way down to tip (Kerala) of the Western Indian Peninsula and displays the common ploidy level of 2n=24. Both morphospecies can survive in either grasslands, where it dies back annually or deciduous or evergreen forests up to 2000 m (Ridsdale 1974). This resilient ecological adaptation may have allowed it to persist in India and Indochina in spite of the Neogene aridification, which could have extirpated other strictly tropical taxa. Phylogeographic analysis of this clade will be able to test the out-of-India hypothesis.

Clade II. This clade evolved in West Malesia during the early Miocene (21 mya) when high sea levels inundated parts of this region followed by dispersals into the Philippines, whose oldest islands were partly uplifted at this time (Hall 1998). The Madagascan *L. spinea* is embedded in clade II implying that this species was derived from Asia via long-distance dispersal since the timing of the split (12 mya) does not fit a Gondwanan vicariance event. This trend has also been illustrated by Renner et al. (2001) in some members of Melastomataceae and by Kulju et al (2007) in the Euphorbiaceae genera *Mallotus* and *Macaranga*, though Kulju et al. (2007) also suggested the possibility of an earlier Eocene-Oligocene dispersal via the Lemurian Stepping stones (Schatz 1996).

Clade III. The ancestral area of this large-flowered clade was placed in West Malesia with subsequent dispersal to Bismarck Archipelago (New Guinea) in the early Miocene (21 mya). The restriction of the earliest-diverging species, *L. amabilis*, to northwest Borneo is expected since the rest of Borneo was under water during this time. The next 'basal' species, *L. macropus,* is endemic to Bismarck Archipelago, which was once part of a continuous arc comprised of east Philippines, northern Moluccas, and the north New Guinea terranes that ran parallel northeast of the New Guinea craton (Hall

1998; Heads 2002). These arc terranes then moved southwest to converge with this craton. It may have been during this time that the West Malesian ancestor dispersed into the Bismarck Archipelago and then invaded New Guinea and the Philippines, where the more derived species are found. A series of complex geological movements in the Miocene (Heads 2002) may have fragmented *Leea* populations stimulating consequent speciations in New Guinea. Meanwhile, a substantial portion of the major islands of the Philippines (Luzon and Mindanao) were already exposed by mid-Miocene (Steppan et al. 2003) which may have been colonized by an ancestral population from New Guinea followed by Pliocene speciations as more islands surfaced.

Clade IV. As discussed in chapters 1 and 2, this clade includes the species complexes of *L. guineensis* and *L. indica*, with other distinct species nested in it. The cryptic evolution of many entities in clade IV belies its wide geographic distribution (i.e. Africa, India, Indochina, Malesia, Micronesia). This clade was shown to have evolved simultaneously in both Indochina and Australia, which were still appreciably separated in the late Oligocene (26 mya). The two major lineages (a vs. b+c+d) of this clade show a vast trans-Malesian disjunction, which may have been an artifact of inadequate taxon and geographic sampling. Inclusion of data from the Malayan-Sumatran *L. simplicifolia*, the Irian Jayan *L. gonioptera*, as well as from other unsampled cryptic species in the intervening regions of Java, Sulawesi, Lesser Sunda islands, and Moluccas may confirm if this is a real disjunction.

Based on available evidence, subclade a's split may have begun when a founding population was stranded in Australia in the late Oligocene (26 mya), isolating it from the rest of the population in West Malesia and Indochina. This population was the source of

species now inhabiting the Solomon and Fiji islands, which were once part of a continuous island arc that developed between early Eocene and early Miocene (Nunn 1998).

The ancestors of subclades b+c+d remained in Indochina with the ancestors of subclades “c” and “d” successfully colonizing and eventually speciating in emergent islands, as the warm and wetter mid-Miocene encouraged rapid diversifications. The endemicity of subclade “b” in Indochina may be explained by the severance of this region from the West Malesian islands towards the end of the Miocene. Subclade “c” includes African/Madagascan species arising from Asian species due to long-distance dispersals, a trend that was also seen in *L. spinea.* Subclade “d” includes taxa found all over Indomalaya and reflects the complex dispersal patterns of a predominantly archipelagic region.

Four smaller clades make up subclade “d”. Subclade d1 evolved in India in mid-Miocene during the Himalayan orogeny and dispersed eastwards when the younger islands of Malesia became emergent. It is evident that Indochina served as a cradle of evolution for d2, d3, and subclade “c”. These lineages show late Miocene split (9-10 mya) between Indochina and West Malesian species which lends support to the inundation of the Thai-Malay border in the Miocene that may have fragmented populations (Woodruff 2003). A dispersal to the Philippines from Indochina also occurred in mid-Miocene (15 mya) with the Philippine clade almost entirely endemic with entities from Taiwan (K) and Palau (L) embedded in it (Fig. 3). A dispersal from northern Philippines to Taiwan had occurred when the latter emerged in the Pliocene

(Hall 1998). The island of Palau, which is only 500 miles east of the Philippines, had also received *Leea* diaspores sometime in the Pliocene.

Clade IV had undergone rapid radiation facilitated by the ecological novelties presented by the Asia-Pacific region in the late Tertiary. Dating estimates suggested that extant *Leea* species are products of Neogene diversifications, possibly encouraged by the dynamic geological events and climatic fluctuations that beset this period (Hall 1998, 2002; Morley 2000). Populations were fragmented repeatedly by high sea stands (c. 100 m above present-day levels), which was often accompanied by warmer wetter weather that cultivated rainforests (Morley 2000; Woodruff 2003).When sea levels subsided, drier climates set in and grasslands replaced rainforests. These recurrent changes with cumulative durations of c. 12 myr in the Neogene promoted diversifications in Southeast Asia rather than the Pleistocene glaciations (Cannon and Manos 2003; Woodruff 2003; Muellner et al. 2008). Their impact is also reflected in the development of two predominant ecotypes in modern *Leea*--savannah-adapted (clade I, *L. macrophylla, L. compactiflora*) and rainforest-adapted species of clades II and III. Sheldon (1996) asserted that morphological stasis should happen in unstable changing environments, which select for generalist phenotypes that are well-adapted to subsequent fluctuations. This seems to be true for clade IV, in which cryptic speciation coupled with rapid radiation, as manifested in the very short branch lengths (Fig. 3), had occurred. Additional molecular evidence and geographic sampling are warranted to clarify the complex biogeographical patterns within this cryptic but genetically diverse group.

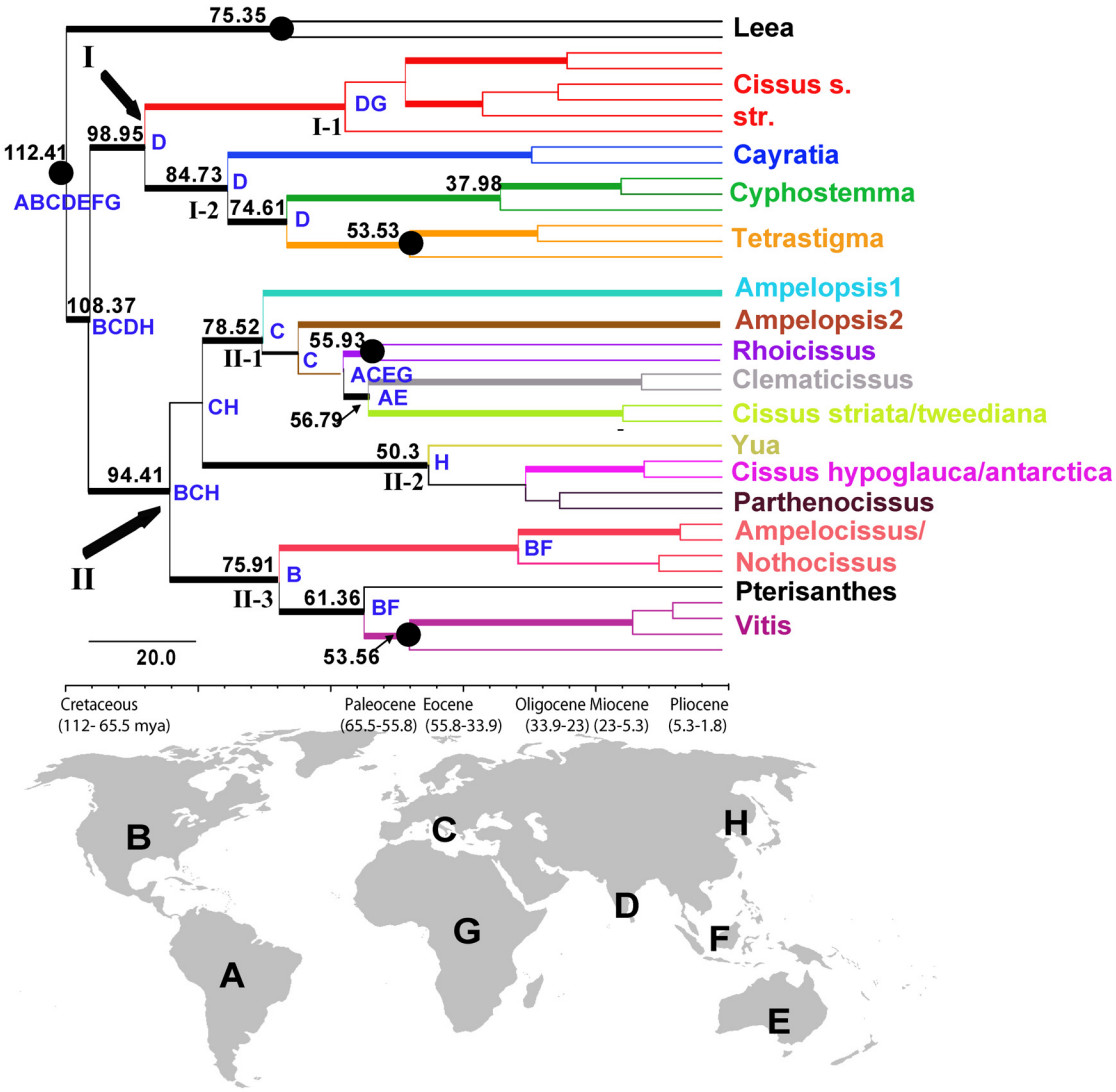

Fig. 2. Vitales phylogeny resulting from divergence time estimation in BEAST superimposed with inferred ancestral areas. Age estimates are presented above the nodes unless indicated. Nodes overlain with black circles were calibrated with known fossil dates (see Methods). Black solid lines represent nodes supported with ≥90% posterior probability. Two major clades were recovered (I, II). Subclade labels (I-1, I-2, II-1, II-2, II-3) are also indicated. A timeline spanning the Cretaceous and Tertiary (Paleocene-Pliocene) epochs is provided for reference. Ancestral areas (A-H) appear in blue font on the phylogeny and refer to the geographic regions used for area optimization: A. South America; B. North America; C. Europe; D. India; E. Australia; F. Southeast Asia (i. e. Indochina and Malesia); G. Africa; and H. Northeast Asia.

Table 1. Diversity, distribution, and Genbank accession numbers for *GAI1* and *trnLF* spacers of Vitaceae taxa included in the BEAST and DIVA analyses (modified from Table 1 in Soejima and Wen, 2006: 279). Each genus may have been represented by more than one species. Species epithets are indicated in the last column followed by the accession numbers. Missing sequences are indicated by --.

Genus	Number of species	Distribution	Genbank accession numbers (species epithet: *GAI1, trnLF*)
Ampelocissus Planch.	95	Tropical Africa, Asia, and Australia with 4 species in Central America and the Caribbean	*elegans:* EF141197, AB234981 *javalensis:* --, AB234984 *polystachya:* EF141198, AB234987
Ampelopsis Michx.	25	Temperate to subtropical Asia, North and Central America (3 spp.), West Asia (2 spp.)	*aconitifolia:* EF141200, AB234989 *albiporcata:* EF141221, -- *cantoniensis:* EF141203, AB234995 *hypoglauca:* EF141214, AB235000 *glandulosa-hancei:* EF141208, -- *glandulosa-heterophylla:* EF141210, --
Cayratia Juss.	63	Tropical and subtropical Asia, Africa, Australia, and the Pacific Islands	*mollisima:* EF141218, AB235003 *pedata:* EF141219, AB235005
Cissus L.	350	All tropical regions with a few in the temperate zone	*anisophylla:* EF141222, AB235010 *antarctica:* --, EF179079 *discolor:* EF141228, AB235011 *elongata:* EF141243, -- *hypoglauca:* --, EF179082 *striata:* --, AB235018 *trifoliata:* EF141232, AB235014 *tweediana:* --, EF179089
Clematicissus Planch.	2	Western Australia	*angustissima:* --, EF179091 *opaca:* --, EF179092
Cyphostemma (Planch.) Alston	200	Mainly in Africa and Madagascar with a few species in India and Sri Lanka extending into Thailand	*maranguense:* EF141257, -- *montagnacii:* EF141258, AB235027 *simulans:* EF141259, --
Nothocissus (Miq.) Latiff	5	Peninsular Malaysia, Sumatra, Bangka, Borneo, and Papua New Guinea.	*spicifera:* --, AB235029
Parthenocissus Planch.	15	12 spp. in E Asia, 1 in India and 3 in North America.	*tricuspidata:* EF141271, AB235043 *quinquefolia:* EF141267, AB235037
Pterisanthes Blume	20	Malay Peninsula, Borneo, Sumatra, Java, Philippines, and peninsular Thailand.	*stonei:* EF141272, AB235046
Rhoicissus Planch.	12	Tropical and South Africa	*digitata:* EF141273, AB235047 *rhomboidea:* EF141275, AB235048
Tetrastigma (Miq.) Planch.	95	Tropical and subtropical Asia, 5 spp. in Australia	*sp:* EF141279, -- *triphyllum:* EF141282, -- *voinierianum:* EF141283, --
Vitis L.	60	Temperate regions of the northern hemisphere, 1 sp. in South America.	*aestivales:* EF141286, EF179095 *flexuosa*: EF141288, AB235077 *thunbergii:* EF141296, AB235082 *vinifera:* AF378125, EF179097
Yua C. L. Li	3	Subtropical China, India (Assam) and central Nepal	*austro-orientalis:* EF141297, AB235086

Table 2. *Leea* taxa included in the BEAST and DIVA analyses along with their voucher information (cf. Table 1 of chapter 1)

Taxon names	Geographic distribution	Accession numbers
Leea aculeata Bl. ex Spreng.	West Malesia, Philippines	*MO19*
Leea acuminatissima Merr.	Philippines	*FEs.n., WE8242*
Leea aequata L.	Indomalaya	*DA99069,KE3080, WE7494, YA4*
Leea amabilis Veitch	Borneo	*AR9415*
Leea angulata Korth ex Miq.	West Malesia, Philippines	*WE10230*
Leea arborea Sieber ex Bojer	Mauritius	*LO2647*
Leea asiatica (L.) Ridsdale	India, Indochina	*AV2190, WE9036*
Leea aspera Wall.	India	*RA576, SU9480014*
Leea coccinea Planch.	Myanmar	*RI264*
Leea compactiflora Kurz	India, Indochina	*RH55103*
Leea congesta Elmer	Philippines	*MO3*
Leea coryphantha Laut.	New Guinea	*HO10688*
Leea cumingii Clarke	Philippines	*MO8*
Leea cuspidifera Baker	Madagascar	*GE62078*
Leea guineensis G. Don	New Guinea, tropical Australia, Fiji, Solomon Islands	*DG3177, JA2622, RE705, SM7773, TA4316, TA4320*
Leea heterodoxa K. Sch. & Laut.	New Guinea	*HE1583*
Leea indica (Burm.f.) Merr.	India	*FE2031, NI2995*
Leea longifoliola Merr.	Tropical China	*CH78319*
Leea luzoniensis Elmer	Philippines	*LI1206, MO31*
Leea macrophylla Roxb. ex Hornem.	India, Indochina	*WE7415*
Leea macropus K. Sch. & Laut.	Bismarck Archipelago (New Guinea)	*TA16698*
Leea maculata Desf.	Tropical Africa	*AL7506, GE5851, GO119*
Leea magnifolia Merr.	Philippines	*ED3509*
Leea monticola Desc.	Madagascar	*WE9569*
Leea negrosense Elmer	Philippines	*MO32, MO37, WA6727, YA7, YAs.n.*
Leea palanensis n. sp.	Philippines	*MO6*
Leea papuana Merr. & Perry	New Guinea	*KA1339*
Leea philippinensis Merr.	Philippines, Taiwan	*MO17, YAs.n.*
Leea quadrifida Merr.	Philippines	*USC821*
Leea robusta Roxb.	India, Indochina	*WE7417*
Leea rubra Bl. ex Spreng.	Indomalaya, tropical Australia	*LE127, PU6703*
Leea setuligera Clarke	India, Thailand, China	*CH3311*
Leea spinea Desc.	Madagascar, Comoros	*BA646*
Leea sumatrana Miq.	West Malesia, Philippines	*BO2438, LE126, RA818, WE8341, WE10237*
Leea zippeliana Miq.	New Guinea	*SC4387*
Leea n. sp.1	Myanmar	*KR975877*
Leea n. sp.2	Myanmar	*KR37301*
Leea n. sp.3	Palau	*CA696*
Leea n. sp.4	Thailand	*AN5149, MA90692*

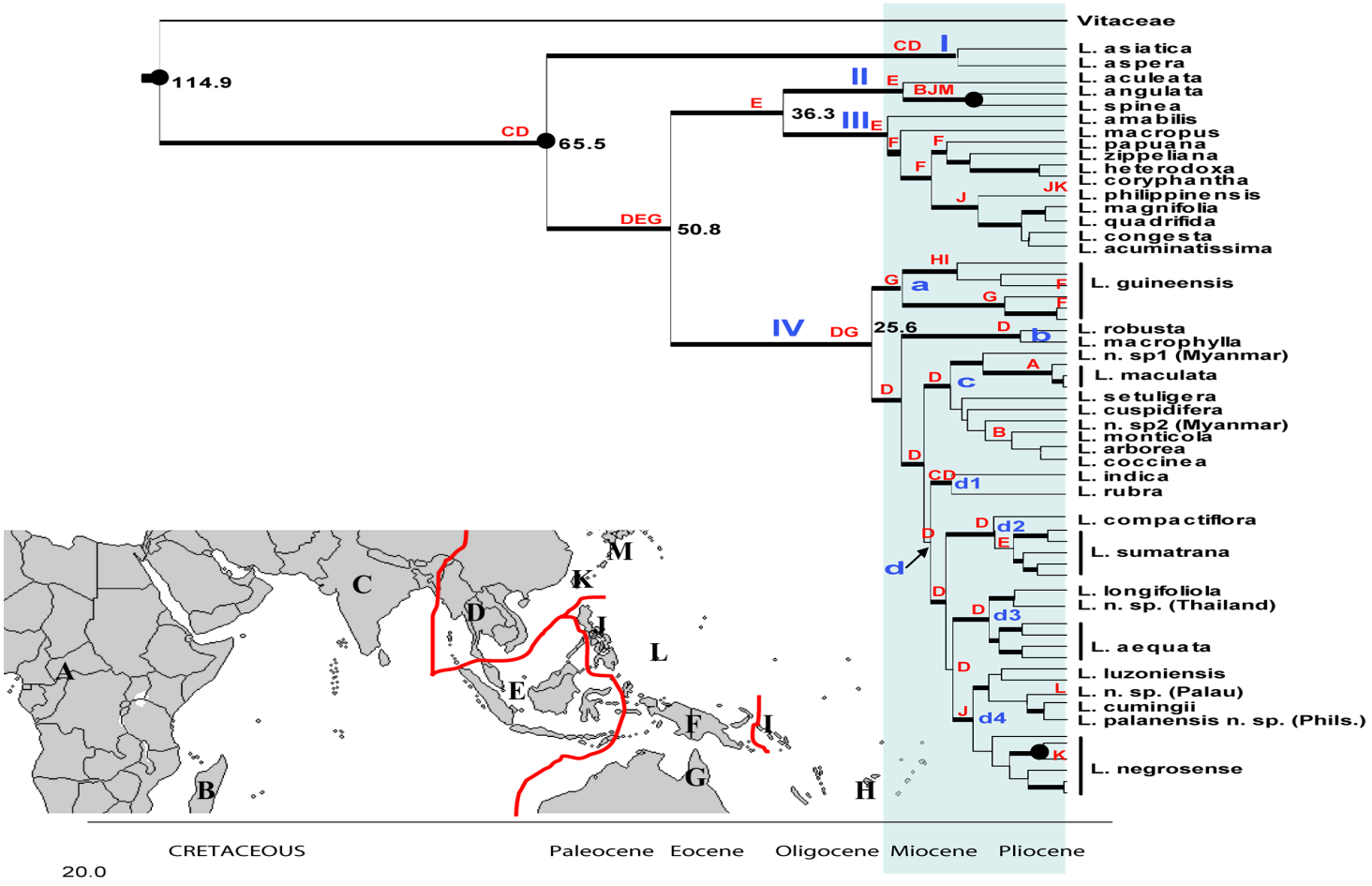

Fig. 3. Phylogeny of *Leea* with date estimates and putative ancestral areas (A-M, red text; refer to map, inset) as inferred from BEAST and DIVA analyses. Nodes with black circles were calibrated using fossil dates (see Methods). Nodes with no ancestral areas depicted have the same areas as the labeled immediate common ancestor. Major clades (I, II, III, IV) and subclades (a, b, c, d including d1, d2, d3, d4) of clade IV are labeled. Black solid lines represent nodes with ≥90% posterior probability. Light blue box refers to the Neogene period (Miocene, Pliocene) when most of *Leea* diversifications occurred.

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PUBLICATIONS

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