EVOLUTION AND SYSTEMATICS OF THE ANGIOSPERM ORDER GENTIANALES WITH AN IN-DEPTH FOCUS ON LOGANIACEAE AND ITS SPECIES-RICH AND TOXIC GENUS *STRYCHNOS*

by

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A Dissertation Submitted To The

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Plant Biology

Written under the direction of

Dr. Lena Struwe

And approved by

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New Brunswick, New Jersey

October 2008

ABSTRACT OF THE DISSERTATION

Evolution and Systematics of the Angiosperm Order Gentianales with an In-depth Focus on Loganiaceae and its Species-rich and Toxic Genus *Strychnos*

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The Gentianales includes five families known for their horticultural value and medicinal effects: Apocynaceae (milkweeds and rosy periwinkle), Gelsemiaceae (Carolina jessamine), Gentianaceae (gentians), Loganiaceae (strychnine plants), and Rubiaceae (coffee and quinine). They are a monophyletic assemblage, but the relationships between the families have been uncertain. The Loganiaceae are a mostly pantropical group with a few members reaching into temperate North America and Australia. This family includes 15 genera that have been divided into four tribes, one of which, Strychneae, was paraphyletic in previous analyses based on chloroplast and morphological data. *Strychnos* is the largest genus in Loganiaceae with approximately 200 species distributed throughout the tropics. This genus is well-known for its alkaloid production, in particular that of strychnine. Although strychnine has been popularized for its potential nefarious uses, many *Strychnos* species have been lauded for their medicinal properties conferred

by other compounds. *Strychnos* was divided into 12 sections based on morphology, but the monophyly of these was in doubt as the sections were morphologically, chemically, and anatomically heterogenous. Combined analyses of four chloroplast regions, *mat*K, *ndh*F, *rbc*L, and *trn*L, placed Rubiaceae as the most basal family in the Loganiaceae. Loganiaceae and Gelsemiaceae were the two subsequently diverging clades, and Apocynaceae and Gentianaceae were sisters in the most nested position. Within the Loganiaceae, tribe Antonieae was basal to all other tribes. Strychneae was resolved as monophyletic only when morphology was included in a combined analysis with a nuclear ribosomal gene region (ITS) and *rps*16 of the chloroplast genome. An analysis of the secondary structure of the ITS region resulted in possible new synapomorphies for tribes Antonieae and Spigelieae within the Loganiaceae. The current sectional treatment of *Strychnos* does not reflect the evolution of the genus, and recommendations for improving the classification will be made. This work will have large implications for the understanding of chemical and morphological evolution on ordinal, familial, and tribal levels in the Gentianales, and provides a framework for further studies in biogeography, habit evolution, and speciation processes.

ACKNOWLEDGEMENTS

There were times where the temperature was just right and the sky was just blue enough that I wanted to leave my dissertation behind. Thanks to my friends and family that assured me that there were better days ahead and encouraged me to stay focused. You were right.

My greatest appreciation goes out to my advisor, Lena Struwe, who guides her students along the path from novice to scholar with compassion and perseverance. Thanks also to my committee for their support and expertise. And without my labmates, my entire graduate experience would have been lackluster, to you I extend my utmost gratitude. One cannot forget the staff of the Plant Biology and Pathology Department; I would still be cursing the photocopier without you.

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INTRODUCTION

The Gentianales is an order with more than 16,000 species in more than 1,500 genera (Stevens, 2001 onwards). This accounts for approximately 6% of all flowering plants. Five families are included in this order: Apocynaceae (milkweeds), Gelsemiaceae (jessamines), Gentianaceae (gentians), Loganiaceae (*Strychnos* and relatives), and Rubiaceae (coffees). Rubiaceae is the largest family with approximately 11000 species, followed by Apocynaceae with 4500 species, and Gentianaceae with 1650 species. Loganiaceae and Gelsemiaceae are much smaller families with 400 (Struwe et al., In press) and 11 (Jiao et al., 2007) species, respectively, in a total of 17 genera.

Gentianales has been recognized as a monophyletic unit (Olmstead et al., 1992; Backlund et al., 2000; Bremer et al., 2004), but the relationships between the families are unclear. Generally, unresolved trees were produced by analyzing the chloroplast regions *matK*, *ndhF* and *rbcL* in conjunction with each other (Jiao et al., 2007), and the *trnL* region alone (Thiv et al., 1999). However, a phylogenetic analysis of the Gentianales using the *ndhF* and *rbcL* gene regions, but with a broader sampling than that of Jiao and Li (2007) yielded a tree well-resolved at the family level (Backlund et al., 2000). The Rubiaceae were placed sister to all other Gentianales families, with Gentianaceae and Loganiaceae as the next diverging clades. Apocynaceae and Gelsemiaceae were sisters in the most nested position. The sampling for the Backlund et al. (2000) work included 35 genera from the order, but was missing representatives from some major lineages in the Apocynaceae and Gentianaceae.

Comprehensive datasets of the *rbcL*, *ndhF*, *matK*, and *trnL* regions of Gentianales genera have been gathered and deposited in GenBank (chapter 1). By drawing from this resource, a dataset that included all major lineages of each of the Gentianales families was assembled. Additional sequencing for the Loganiaceae and Gentianaceae was done to produce a final collection of *matK*, *ndhF*, *rbcL*, and *trnL* sequences that included all Apocynaceae and Rubiaceae subfamilies, all Gentianaceae and Loganiaceae tribes, and both Gelsemiaceae genera for a total of 49 Gentianales genera. Nine outgroups were included from the Cornales, Dipsacales, Solanales, and Lamiales. The *trnL* region was the most variable, so the secondary structure of the sequences was taken into account during the alignment process. Bayesian analyses were conducted on the datasets individually and combined.

The Rubiaceae were placed basal to all other Gentianales families in the combined analysis (chapter 1), which is in agreement with the works of Backlund et al. (2000) and Jiao and Li (2007). The Loganiaceae and the Gelsemiaceae were the next diverging clades, but with low branch support. The Apocynaceae and Gentianaceae were sisters in the most nested position. The Gelsemiaceae are a relatively new family (Struwe et al., 1994), and the constituents have a history of being placed inside the Loganiaceae (Bentham, 1856) or the Apocynaceae (Endlicher, 1841) based on morphological features. The intermediate position of the Gelsemiaceae between the Loganiaceae and the Apocynaceae is, therefore, not surprising.

The Gentianales phylogenetic study set up the opportunity to conduct a molecular dating analysis calculating ages of the different families in the order. Numerous Rubiaceae fossils have been reported and a compilation of these was recently produced

by Graham (Accepted). The fossils were subjected to a verification process that rated them as “accepted”, “pending”, and “NA” (chapter 1). Only those fossils that were “accepted” and linked to genera present in the dataset were used as calibration points in a dating analysis performed in BEAST. One fossil from the Apocynaceae (*Periploca*; Muller, 1981) and one fossil from the Gentianaceae (*Lisianthius*; Graham, 1984) were used as additional fossil calibration points. Two putative Loganiaceae fossils, *Strychnos* (Chaney et al., 1933) and *Geniostoma* (Wood, 1956), were investigated, but found unsuitable to include in this study due to incomplete preservation or the inability to verify their identity due to damage. In total, four fossils were used for the molecular dating of the Gentianales, which were estimated to have diverged in the late Jurassic or early Cretaceous, and most of the families were estimated to be from the early Paleocene to the middle Miocene. Experimentation with multiple prior distributions (uniform, log normal, exponential) always resulted in broad confidence intervals and age estimates older than those from previous analyses, therefore, these results should be viewed critically.

A more in-depth review of the Loganiaceae was conducted (chapter 2). This Gentianales family has had varying circumscriptions that range from one genus (Taktajhan, 1997) to 29 (Leeuwenberg et al., 1980). Currently, the Loganiaceae has 15 genera (Dunlop, 1996; Struwe et al., In press) divided into four tribes: Antonieae, Loganieae, Spigelieae (monogeneric), and Strychneae. Strychneae includes three genera, *Gardneria*, *Neuburgia*, and *Strychnos*. The first two genera are Asian endemics and have few species. *Strychnos*, on the other hand, is the most-speciose genus of the family with approximately 200 species pantropically distributed (Krukoff et al., 1942; Leeuwenberg et al., 1980). The relationships of the genera in Strychneae have been difficult to

characterize as their placement in gene trees vary. The tribe seems to be an artificial grouping, and was paraphyletic in a study using two chloroplast genes, *ndhF* and *rbcL*, due to the inclusion of Spigelieae (Backlund et al., 2000).

Phylogenetic analyses of two faster evolving genes, *rps16* and the internal transcribed spacer (ITS), were conducted in an effort to tease apart the relationships between genera in the Loganiaceae with an emphasis on Strychneae (chapter 2). Twelve out of the 15 Loganiaceae genera were sequenced, including the poorly-known *Norrisia* of tribe Antonieae. This is the first publication of sequence data for this under-studied Asian genus. A morphological analysis was also conducted in an effort to place two segregates of *Mitrasacme*, *Phyllangium* and *Schizacme* (Dunlop, 1996), which had no sequence data, into context. *Usteria* was also missing sequence data due to the lack of suitable quality material for DNA extraction.

The ITS region is from the nrDNA, and has become a staple of phylogenetic analyses for plant groups. The variability of the primary structure in Loganiaceae made it valuable for a phylogenetic study, but the region is prone to indels complicating its alignment between genera (Baldwin et al., 1995). However, the secondary structure of the molecule is highly conserved, for example, similarities between flowering plants and algal structures for the second half of the ITS region have been reported (Mai et al., 1997). Additionally, Goertzen et al. (2003) developed a minimal secondary structure model of the entire ITS region conserved among members of the Asteraceae.

The secondary structure of the Loganiaceae ITS region was estimated by folding sequences in RNAstructure (Mathews et al., 2006) and using these results to develop a constraint file for an alignment program, RNAsalsa (Misof et al., Submitted), to use as a

guide in folding all the Loganiaceae sequences (chapter 2). Compensatory base changes were viewed as additional evidence to support the presence of stems. The secondary structure of Loganiaceae was similar to that of Asteraceae, both asterids, but had an additional stem in ITS1. Loganiaceae's structure was nearly identical to the consensus structure of Gentianaceae, also from the Gentianales (Molina et al., Accepted pending revision). Some trends were evident at the tribal level as Antonieae was lacking or had a truncated stem 1A and Spigelieae had an abridged stem 2D.

The *rps16* data is from the chloroplast genome and provided an independent data source from which to estimate Loganiaceae's phylogeny (chapter 2). Antonieae was used to root the trees as this tribe was consistently basal to the others in the ordinal level studied conducted for this dissertation. The topologies of the ITS and *rps16* trees were different, but both supported the monophyly of Antonieae and Loganieae. Strychneae was not monophyletic and Spigelieae's position was variable.

Strychneae was only supported in the morphological analysis of the family that included 58 characters. A synapomorphy for Strychneae is indehiscent fruits, but the fruits are different types. *Neuburgia* and *Gardneria* have drupes and *Strychnos* has berries (Leeuwenberg et al., 1980). If this character is removed from the analysis, Strychneae is no longer monophyletic. If all the evidence is combined, the resulting trees support the present tribal classification (Struwe et al., In press), Strychneae included. Although Strychneae is supported, the author feels that the consistent failure of molecular data to resolve Strychneae as a monophyletic group and the tribe's reliance on a single synapormorphy that may be a product of parallel evolution suggests that this tribe is not a

natural group. Resurrection of tribe Gardnerieae Endl. (Endlicher, 1838) should be considered.

Strychnos is the most speciose genus in Loganiaceae with approximately 200 species that grow as lianas, shrubs, or small trees throughout the tropics (Hill, 1917; Leeuwenberg, 1969; Krukoff, 1972). *Strychnos* is most well-known for its production of alkaloids, such as the infamous strychnine. Additionally, the genus has a history of use as traditional medicines to treat fevers (Bisset, 1970), parasitic infections (Burkill et al., 1995), malaria (Rafatro et al., 2000), and many other conditions.

A large-scale phylogenetic analysis of *Strychnos* was conducted for the first time including approximately 50% of the species spanning its geographical distribution (chapter 3). The monophyly of *Strychnos* was in doubt as the genus is morphologically variable and was paraphyletic in a study using morphological, anatomical, and chemical characters (Struwe et al., 1994). The genus was divided into 12 sections (Leeuwenberg et al., 1980), but the monophyly of these is also doubtful as the descriptions are highly heterogeneous.

The ITS region was sequenced for 102 *Strychnos* species and analyzed using Bayesian and maximum parsimony methodologies (chapter 3). *Strychnos* was monophyletic, but the sections were not, with one exception - *Spinosae*. This is an African section with four species which share distinct morphological characters such as the presence of spines and deciduous stipules, and lack interxylary phloem. The remaining sections were polyphyletic. Generally, species grouped based on geography, and this supports the older system of preparing sectional treatments that was used by Duvigneaud (1952), Hill (1917), and Krukoff (1942; 1972), which focused on the genus

one continent at a time. This resulted in a higher number of monophyletic sections with more precise morphological descriptions. The overarching work of Leeuwenberg and Leenhouts (1980) was valuable in putting many *Strychnos* species from each continent into a broad evolutionary context, but did not enhance the systematic knowledge of the genus by forcing African *Strychnos* species into sections with American and Asian species

It is recommended that section *Strychnos,* which includes Asian and American species, be dissolved, and Progel's (1868) *Longiflorae* be reinstated (chapter 3). A new section, for the Asian species that were included in *Strychnos* sensu Leeuwenberg and Leenhouts (1980) be initiated. This section, since it includes the type species, *Strychnos nux-vomica*, of the genus would have to be named *Strychnos*, but this section would be equivalent to Hill's *Tubiflorae* (1917). An additional recommendation includes a new section for a group of American species that was briefly suggested by Krukoff and Monachino (1942) and subsequently supported by the ITS data that includes *Strychnos chlorantha, S. colombiensis, S. panurensis, S. jobertiana,* and *S. ramentifera*.

Section *Breviflorae*, which initially only included American species (Progel, 1868), was divided into two subsections using characters of the testa (Krukoff et al., 1969). However, the subsectional division was never recognized in subsequent treatments by other authors, so when African species were added to the section (Leeuwenberg et al., 1980) they were never assigned a subsection. The ITS data supports the subsectional division of the American species with the exclusion of a portion of the species. The inclusion of the African species resulted in this section being one of the

most scattered amongst the branches of the ITS phylogenetic tree, and it is recommended to remove them from *Brevilforae*.

Investigation of the ITS data revealed polymorphisms that seemed to heritable, and cytological studies on the genus (Gadella, 1980) identified numerous *Strychnos* polyploids. The combination of these two facts made it advisable to investigate the possibility of hybridization amongst *Strychnos* species.

ITS is biparentally inherited and is a high-copy number nuclear gene region, so hybridization can have three main results: both parental sequences represented as polymorphic peaks in the sequence chromatogram (Soltis et al., 1991), formation of chimeric sequences (Nieto Feliner et al., 2004), or loss of one of the parental ribotypes (Fuertes Aguilar et al., 2003). Chromatograms were scanned by eye for polymorphisms that were present in both the forward and reverse sequencing reactions (chapter 4). All polymorphisms were recorded, but only those that were shared by multiple accessions were used for drawing conclusions on hybrid ancestry.

An Asian subclade of *Strychnos* species shared multiple polymorphisms, suggesting that they may have a common hybrid ancestor. The accumulation of additional polymorphisms in subsequent branches suggests that hybridization may be ongoing. Using Splitstree (Huson et al., 2006) to view the ITS data as a network showed that this portion of the tree had many competing topologies. These species are in a relatively derived position in the tree and it is possible that they are a sight of active evolution and that reproductive barriers are leaky. This hypothesis can explain the difficulties in characterizing two members of this subclade, *Strychnos axillaris* and *S. minor*, which have more than 25 synonyms each.

The cultural significance of *Strychnos* to tropical traditional peoples has drawn the attention of many researchers. This work made significant progress in understanding this large, widespread, and variable genus and will be useful in guiding future chemical, biogeographic, and taxonomic studies. On a broader scale, progress was also made in assessing the current tribal classification of Loganiaceae. It is suggested that Strychneae is an artificial group, but additional research should be done to determine if it is appropriate to reinstate tribe Gardnerieae, and the placement of Spigelieae remains unclear. A more complete chloroplast dataset representative of all major Gentianales lineages was obtained and a Cretaceous age estimate for the order was corroborated.

A molecular dating analysis using the non-parametric rate smoothing (NPRS) method should be conducted to compare the results of the different methodologies for the datasets presented in chapter 1. The dates from this analysis will be valuable in presenting biogeographic hypotheses for Loganiaceae and *Strychnos*. The relationships between genera in Loganiaceae have been complicated by ancient hybridization events (chapter 4) and further research into this subject would be valuable to clarifying the placement of Strychneae and Spigelieae. Within *Strychnos*, ample opportunity exists for investigating various aspects of the genus' wood anatomy. In the rays of some species are 'holes' (personal observation) that appear similar to "breakdown areas" that were found in Myrsinaceae (Lens et al., 2005). Other discoveries that have been noted are the presence of faint helical thickenings in the vessels of a sample of *Strychnos spinosa*, a tropical savanna species, and the absence of vestures in the vessel pits of the same sample (personal observation). This provides evidence in support of the hypothesis that vestures and helical thickenings may have similar functions (Carlquist, 1982; Jansen et al., 2000).

A final recommendation for future studies is to work with potential species complexes, such as *Strychnos brasiliensis, S. minor, S. axillaris,* and *S. angolensis,* from a population genetic perspective.

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CHAPTER 1

Phylogeny of the Gentianales (Asteridae)

Abstract

The Gentianales include five families: Apocynaceae, Gelsemiaceae, Gentianaceae, Loganiaceae, and Rubiaceae, but the relationships between them are still uncertain. Sequences from four chloroplast regions, *matK*, *ndhF*, *rbcL*, and *trnL* were analyzed separately and combined using a Bayesian framework and the phylogenetic result subsequently used to perform a molecular dating analysis. The Gentianales are monophyletic as well as all currently recognized families within the order. In the analysis of the combined data the Rubiaceae were placed sister to all other Gentianales families, with Loganiaceae and Gelsemiaceae being the next subsequent diverging clades. Finally, The Apocynaceae and Gentianaceae were sister groups in the most nested position. Molecular dating using four fossil calibration points suggested divergence dates much older than previous estimations, but with very broad ranges whose lower ends are similar to previous studies. Based on this methodology, the Gentianales were estimated to have diverged during the late Jurassic to the early Cretaceous, with most of the families estimated to be from the early Paleocene to the middle Miocene. This result is not in concordance with fossil records from deeper clades in the Asteridae, and should be evaluated critically in further analyses since it appears that the method potentially overestimates ages.

Introduction

The Gentianales have been the subject of numerous investigations, but the exact familial relationships remain elusive. A consensus classification for the order was assembled from publications dating from the 1960s to the early 1990s by Nicholas and Baijnath (1994). This included the works of Benson (1979), Cronquist (1988), Dahlgren (1983), Goldberg (1986), Heywood (1978), Melchior (1964), Stebbins (1974), Takhtajan (1983), and Thorne (1992). The effort involved in developing this consensus classification is a reflection of the variability in the opinions of previous researchers on the evolutionary history of this group; however, Nicholas and Baijnath noted that the reviewed works showed a trend towards agreement. The consensus classification resulted in six families in the Gentianales: Apocynaceae, Asclepiadaceae, Gentianaceae, Loganiaceae, Rubiaceae, and Saccifoliaceae.

Many of the classifications reviewed for the consensus publication were written prior to the mainstream usage of sequence data and phylogenetic methodology advances. Since then, Asclepidaceae has been reduced to a subfamily of Apocynaceae (Endress et al., 2000) and a new family, Gelsemiaceae, was described by Struwe et al. (1994) as a segregate from Loganiaceae. Saccifoliaceae was a monotypic family consisting of the relatively recently discovered genus *Saccifolium* (Maguire et al., 1978), but it has been reduced to a tribe in Gentianaceae (Struwe et al., 1998; Struwe et al., 2002). Therefore, the current status of Gentianales is five families: Apocynaceae, Gelsemiaceae, Gentianaceae, Loganiaceae, and Rubiaceae.

Molecular studies have provided progress in the phylogenetic concept of the Gentianales, but there are incongruencies in trees resulting from analyses of different

chloroplast genes. For example, Rubiaceae was placed basal to all other Gentianales families using the *ndhF* and *rbcL* genes (Backlund et al., 2000). However, it was in a more derived position in *matK* (Civeyrel et al., 2001) and *rps16* (Andersson et al., 1999) analyses. Trees with very little resolution were produced using the *trnL* region, but the Rubiaceae were again placed in a more derived position (Thiv et al., 1999; Yuan et al., 2003).

A combined analysis of the *matK*, *ndhF*, and *rbcL* regions from three genera in each family was performed recently (Jiao et al., 2007). In a strict consensus tree resulting from a combined analysis of this data, Apocynaceae, Gelsemiacae, Gentianaceae, and Loganiaceae were united at a polytomy into one clade with Rubiaceae as its sister. Large datasets of the *rbcL*, *ndhF*, *matK*, and *trnL* regions of Gentianales genera are available from GenBank. This study unites the work of numerous researchers, providing a cohesive resource for the scientific community. Additionally, new DNA sequences from numerous chloroplast regions (*ndhF*, *rbcL*, *trnL*, and *matK*) were generated to increase taxon sampling in an effort to represent all major lineages in the Gentianales. This dataset has set the stage to perform a molecular dating analysis that has multiple fossil calibration points.

Molecular dating and fossil evidence. A fossil-based minimum age for the Gentianales is 53 million years (mya) (Magallón et al., 1999). Using DNA sequence data to estimate the divergence of the same order with one distant fossil calibration in the Fagales pushed the age of the Gentianales back into the Cretaceous at 83-89 million years ago (Wikström et al., 2001). A more recent estimate uses six fossil calibration points and six chloroplast DNA markers and, generally, added an additional 10-20 million years to

the age of many asterids (Bremer et al., 2004). The latter study was on a broad scale and included many orders represented by one genus each. The patterns of distribution for some families in the Gentianales have prompted the suggestion of an even older origin. For example, members of the subtribe Potaliinae (Potalieae: Gentianaceae) are distributed in the tropics of Africa, Asia, and South and Central America, which may possibly be an echo of the Gondwanic breakup (Struwe et al., 1997). Multiple genera from all five Gentianales families representing most major lineages plus outgroups from the Lamiids and Campanulids of the Euasterids and a Cornales representative were included in this multi-gene study. This publication collates the existing sequence data for the Gentianales, and is the first to perform an in-depth molecular dating analysis focused on the order.

The Gentianales are represented in the fossil record numerous times. Graham compiled a review of Rubiaceae fossil records and rated them as "NA" if the fossil had features that excluded it from the typical bauplan of Rubiaceae or only casual references were made to its familial resemblance, "pending" if more information was needed, and "accepted" if reexamination showed it had Rubiaceae characteristics and its age is not improbable (Accepted). Apocynaceae and Gentianaceae pollen have been recorded from the Paleocene to the later Miocene (Muller, 1981; Graham, 1984a), and there are putative leaf impressions and seed fossils of Loganiaceae (Ettingshausen, 1879; Chaney et al., 1933; Wood, 1956) . The Gelsemiaceae are the smallest family in the Gentianales, and the only one without any described fossils. These fossils records were valuable as calibration points in the molecular dating analysis that was performed for this study.

Materials and Methods

Taxon sampling. Representatives from all five families in the Gentianales were included for every gene dataset, and efforts were made to include the majority of major lineages within each family. All five subfamilies of Apocynaceae and all three subfamilies of Rubiaceae were represented. All four tribes of Loganiaceae and all six tribes of Gentianaceae were included. Gelsemiaceae is a small family of two genera, *Gelsemium* and *Mostuea*, and both were included in this study. Species of the same genera were used interchangeably to represent the genus. There were a total of 58 genera used in this study; for further information on classifications and additional details on the datasets see Table 1.1.

The classifications of Endress and Bruyn's (2000) for Apocynaceae, Struwe et al. (2002) for Gentianaceae, Struwe and Motley for Loganiaceae (In press), and Andersson and Rova (1999) for Rubiaceae were followed. All Apocynaceae, Gelsemiaceae, and Rubiaceae sequences were taken from GenBank (see appendix 1 for accession numbers). A portion of the Gentianaceae and Loganiaceae sequences were also from GenBank and others were generated for this study. Members of the Solanales, Lamiales, Dipsacales, and Cornales were used as outgroups, and *Hydrangea* (Cornales) was used to root the trees based on an analysis of Asterids using six chloroplast markers (Bremer et al. 2004).

Extraction, amplification, and sequencing. The *rbcL* and *matK* primers of Civeyrel and Rowe (2001) plus the *matK* primers of Thiv et al. (1999), the *ndhF* primers of Backlund et al. (2000), and the *trnL* primers of Taberlet et al. (1991) were used. All the primers used to sequence the four chloroplast regions and their respective thermocycling programs are in Table 1.2. The *matK* region was particularly difficult to

obtain for all Loganiaceae and Gentianaceae accessions. Multiple combinations of eleven primers were used to break the region into smaller pieces, yet there were still spans that were too large to obtain from some of the poorer quality herbarium sheets. PCR reactions were prepared as described in Frasier et al. (2008), but with the addition of tetra-methyl ammonium chloride (TMAC) to a concentration of 10μM. Dimethyl sulfoxide was also added to the *matK* reactions to 10% of the final volume.

Alignment and phylogenetic analysis. Separate data matrices were compiled for each gene. MAFFT (Katoh et al., 2002) was used for preparing the alignments, which were then manually adjusted after visual inspection. The *trnL* region forms a secondary structure (Oksanen et al., 2004; Quandt et al., 2005; Taberlet et al., 2007), which was taken into account during the alignment step by using the program RNAsalsa (Misof et al., Submitted). The sequence and secondary structure of *Nymphaea odorata*'s *trnL* intron (Taberlet et al., 2007) was used to create the constraint file for RNAsalsa, which guides the folding process. Minimum free energy values and structures were checked for trends among families or in infrafamilial groups. When appropriate, phylogenetically informative indels were coded using the simple gap coding method of Simmons and Ochoterena (2000).

All phylogenetic analyses were performed with MrBayes v. 3.1 (Huelsenbeck et al., 2001), and the resulting trees were compared. Models were selected according to the Akaike Information Criterion from MrModeltest (Nylander, 2004). The ILD test was used to check for conflicts in the data that would complicate a combined analysis in which each gene region would be isolated into its own partition to permit them to be analyzed according to their most appropriate model.

Molecular dating. The program BEAST (Drummond et al., 2007) was used for the molecular dating analysis. Using TRACER to review the results of a preliminary run showed that the rates varied across the tree, so a relaxed molecular clock (Drummond et al., 2006) was implemented. For each fossil calibration point a normal prior was selected. The operators were optimized according to the recommendations of the BEAST program after the preliminary BEAST run.

Only the Rubiaceae fossils that were identified as "accepted" in Graham's work (Accepted) were deemed eligible to be used as calibration points for this study. Seven Rubiaceae genera that are in this study also have accepted fossils that range in age from the Eocene to the Miocene and are listed chronologically from oldest to youngest: *Cephalanthus, Gardenia, Guettarda, Rondeletia, Pinckneya, Chiococca,* and *Ixora*. Only *Chiococca*, *Guettarda*, *Ixora*, and *Pinckneya* were used in this study. The first two are from the Cinchonoideae and the second two are from the Ixoroideae; no fossils from the Rubiodieae were used. If all the Rubiaceae fossils were used, there would have been many fossil calibration points crowded into one clade of the tree. Multiple fossil calibrations plus the restrictions on the monophyly of all the Gentianales families, except Loganiaceae, can complicate BEAST's task to estimate a tree. The monophyly restrictions were imposed based on the results of the Bayesian analyses of the DNA sequences. Loganiaceae was paraphyletic in the results of a preliminary *trnL* analysis, so monophyly was not imposed for this family.

Two Apocynaceae genera in this study have fossil pollen records, *Alstonia* and *Periploca*, from the upper Miocene and middle Miocene, respectively (Muller, 1981). The *Periploca* fossil was used in the study. Fossil pollen of the gentian genus *Lisianthius*

has been recorded from the Eocene (Graham, 1984b) and fossil pollen similar to *Macrocarpaea* have been found from the Paleocene/lower Eocene (Crepet et al., 1981). References to two Loganiaceae fossils were followed up on by the authors. The first was a possible leaf impression of a *Strychnos* species found in Oregon, USA, that was dated to the early Tertiary (Chaney and Sanborn, 1933). A high quality digital image of this fossil was provided by the University of California, Berkeley, Museum of Natural History. The second fossil was a *Geniostoma* leaf impression from New Zealand dated to the Oligocene (Wood, 1956), and was retrieved for imaging from the collections of the New Zealand Geological Society.

Results

The *matK* matrix included 1684 nucleotides with an additional nine coded indels. The GTR+G model was selected according to the AKAIKE information criterion. The GTR+I+G model was selected for the remaining gene regions (*ndhF*, *rbcL*, and *trnL*). The *ndhF* matrix had 2359 characters with an additional 13 coded indels. The *rbcL* matrix included 1402 characters and no additional indel characters. The *trnL* matrix included 627 characters plus 13 indels and produced a tree with the least degree of resolution - all Gentianales families were placed in a polytomy. The combined matrix had 35 indel characters and 6072 nucleotide characters. The models selected by MrModeltest were applied to each nucleotide partition as appropriate, and the standard model was used for the indel characters.

Outgroups. All trees were rooted with *Hydrangea* (Cornales). Adoxaceae and Valerianaceae of the Dipsacales were placed together on the next most basal branch in

the *matK* (Fig. 1.1A), *ndhF* (Fig. 1.1B), and *rbcL* (Fig. 1.1C) trees. Both the *rbcL* and the *matK* trees placed the Lamiales sister to a clade containing the Solanales and Gentianales. The *ndhF* tree had slightly less resolution than the *rbcL* and *matK* trees as the Gentianales, Lamiales, and Solanales were included in a polytomy. The *trnL* tree differed from the others in that the Solanales were placed as the second diverging clade, followed by the Lamiales, which were sister to the Gentianales (Fig. 1.1D).

Apocynaceae. The Apocynaceae were monophyletic in every analysis, and the relationships between genera were consistent. All subfamilies with multiple representatives were paraphyletic. The Rauvolfoideae were the most basal of the subfamilies, and the Apocynoideae formed the next diverging branches. The resolution of the more divergent terminal branches differed slightly between gene regions. Generally, the Asclepiadoideae were sister to the Secamonoideae, which was included in a polytomy that also had *Apocynum* (Apocynoideae) plus Periplocoideae (Fig. 1.2).

Gelsemiaceae. This family is monophyletic in every analysis, and is always placed in a rather derived position within the Gentianales. Due to the persistence of polytomies between families in these analyses, its sister relationship was uncertain, however it was sister to Loganiaceae in the *trnL* analysis (Fig. 1.1D). The tree resulting from the analysis of the combined data suggested that the Gelsemiaceae was sister to a clade that contains Gentianaceae and Apocynaceae, but with low branch support (Fig. 1.2).

Gentianaceae. The Gentianaceae were monophyletic in every analysis, but their placement varied in all trees. Only in the *trnL* analysis was this family placed sister to all other Gentianales families, otherwise it was always in a more derived position, but the

inclusion of the coded indels caused the collapse of the branch that kept Gentianaceae separate from the other Gentianales families (Fig. 1.1D). In the *matK* analysis, it was the next diverging clade after the Rubiaceae, but was in a polytomy with Apocynaceae and Gelsemiaceae + Loganiaceae in the *ndhF* analysis (Fig. 1.1A-B). The *rbcL* data placed the Gentianaceae in a more derived position in a polytomy with Apocynaceae and Loganiaceae (Fig. 1.1C).

Tribe Saccifolieae were sister to all other Gentianaceae tribes, and Exaceae were placed as the most basal of the remaining tribes in every analysis. Chironeae were the next diverging group. The Gentianeae and Helieae were sisters and placed in a clade with Potalieae (Fig. 1.2).

Loganiaceae. The Loganiaceae were monophyletic in every analysis, except the one based on *trnL* (Fig. 1.1A-C). In this analysis, tribe Antonieae were placed separate from the remainder of the family in a large polytomy (Fig. 1.1D). In the remainder of the tree results, Antonieae were sister to the other three tribes of Loganiaceae: Loganieae, Spigelieae (monotypic), and Strychneae. Strychneae, which includes *Gardneria*, *Neuburgia*, and *Strychnos*, were never monophyletic in this study (Fig. 1.2).

Rubiaceae. The Rubiaceae were monophyletic and placed sister to the other Gentianales families in all analyses except the *trnL* (Fig. 1.1). In this analysis the Rubiaceae were placed within a polytomy that included all the other Gentianales families (Fig. 1.1D). The three subfamilies of Rubiaceae (Cinchonoideae, Ixoroideae, and Rubioideae) were each monophyletic. The Rubioideae were sister to the Cinchonoideae and the Ixoroideae (Fig. 1.2).

Molecular dating. Three BEAST runs totaling 14 million generations were performed, and their results were combined, less the burnin, as the resulting trees and posterior probabilities were similar. The Gentianales were estimated to have diverged 143 mya, with a range of 111-175 mya. The estimated times to the most recent common ancestors of the Apocynaceae were 69 mya (range = 54-84 mya), the Gelsemiaceae were 44 mya (range = 16-81 mya), the Gentianaceae were 88 mya (range = 67.5-107 mya), the Loganiaceae were 81 mya (range = 55-125 mya), and the Rubiaceae were 105 mya (range = 75-136 mya). All estimates had broad confidence intervals (Tab. 1.3, Fig. 1.3).

Discussion

The data presented here support the monophyly of the Gentianales, as well as the monophyly for all families within the order according to their current circumscription. However, the individual gene trees in this work generally had less resolution than those published by other authors, such as Backlund et al. (2000) and Jiao and Li (2007); however, only the *matK* tree is in conflict with previous results (Fig. 1.1A). In the *matK* tree of Jiao and Li, Gentianaceae was placed sister to Loganiaceae, Apocynaceae, and Gelsemiaceae, while here it was placed sister to only the Apocynaceae. This topology is weakly supported by both analyses-less than 50% bootstrap in Jiao and Li, and 76% pp here. This could be a result of the different taxon sampling and different methodologies for inferring phylogenies (maximum parsimony versus likelihood).

The most variable gene region was *trnL*. The Gentianaceae tended to have a shorter *trnL* region, but still maintained the conserved sequence motifs of the secondary structure, such as the P4, P7, R_1, R_2, and S regions (Quandt and Stech, 2005; Taberlet et

al., 2007). A further analysis of *trnL* sequences from the Gentianaceae may reveal interesting patterns in deletion events. A review of the MFE values showed that tribes Saccifolieae and Exaceae of Gentianaceae were higher than in all other tribes in the family, with the exception of *Swertia* (tribe Gentianeae).

The Rubiaceae were placed basal to the rest of the Gentianales with high support. This is in agreement with Backlund et al. (2000), Bremer et al. (2004), and Jiao and Li (2007). The only disagreement with the combined tree of Backlund et al. and here is the placement of Gentianaceae. The difference between the tree from Bremer et al. and here is that Gelsemiaceae and Loganiaceae are sisters instead of subsequently diverging clades, and there is no conflict between the combined tree of Jiao and Li and here.

The relationships in the combined tree reflect the distribution of indole alkaloids. These compounds are present in all but the Gentianaceae. The analysis of the combined data place Gentianaceae in one of the most-derived positions within the order, suggesting that this character persisted in the most recent common ancestor of all the families until its relatively late loss in Gentianaceae (Fig. 1.2).

Molecular dating. It has been shown that narrow taxon sampling can result in overestimating the ages of lower nodes and underestimating the ages of higher nodes in molecular dating analyses with less than 150 taxa (Linder et al., 2005). With this in mind, it was advisable to increase the taxon sampling for the Gentianales beyond that already done. Jiao and Li (2007) focused on Gelsemiaceae, and included twelve genera from the other families. The sampling of Backlund et al. (2000) is only slightly less than in this work for the Gentianales, but was lacking *matK* and *trnL* data. However, increasing the sample size to 150 or more, would have resulted in large amounts of

missing data that would compromise the integrity of all phylogenetic analyses and age estimations.

There were ten fossils that were considered for possible calibration points, seven from the Rubiaceae (Graham, Accepted), two from Apocynaceae (Muller, 1981), and one from Gentianaceae (Graham, 1984a). A fossil flower of the extinct genus *Pistillipollenites* from Texas had similarities to the *Macrocarpaea* of the Gentianaceae (Crepet and Daghlian, 1981), but was later deemed not to be related to this family (Struwe et al., 2002). The inclusion of all the fossil data in the dating analyses pushed the computational abilities of available computers to their limits. Therefore, the older fossils found for each lineage were included in an effort to prevent the underestimation of higher nodes that has been reported to occur in smaller analyses (Linder, Hardy, and Rutschmann, 2005).

There are three records for Loganiaceae fossils. A *Strychnos* leaf impression described by Chaney and Sanborn (1933) was found in Oregon, USA, and dated to the early Tertiary. This date is earlier than the dates for other Gentianales fossils, but is not improbable. This fossil would have been a valuable clue to understanding the genus' biogeographic history as it could have supported the Boreotropical hypothesis (Lavin et al., 1993) of migration for this group. Although the fossil had the typical arcodromous venation pattern associated with *Strychnos* and faint reticulate tertiary veins were also present, it was missing its apex and base. The leaf showed no characteristics that are indicative of *Strychnos*, nor any characteristics that exclude it from *Strychnos*. Therefore, according to the ranking system used in Graham (Accepted), this fossil would be classified as "pending", and was not used for calibration. *Strychnos* seeds were reported

from the fossil flora of Sheppey, England (Ettingshausen, 1879), but could not be examined by the authors so were dismissed for the purposes of this study.

A *Geniostoma* leaf impression from New Zealand was held by the New Zealand Geological Society. It was dated to the Oligocene, which is a reasonable age when compared to the dates for fossils of the Gentianaceae, Apocynaceae, and Rubiaceae. Wood (1956) referenced block 32.1 when mentioning this fossil; however, block 32.1 has no visible leaf impressions or other fossils on it. Blocks 32.2-32.5 were also imaged and reviewed for possible *Geniostoma* leaves, but none could be identified with any certainty as belonging to the genus. Block 32.1 was quite small and it has been suggested that the block was broken, and the location of the portion with the leaf impression is unknown.

As the number of fossil calibrations in molecular analyses increased, so did the age of the asterids (Wikström, Savolainen, and Chase, 2001; Bremer, Friis, and Bremer, 2004). This study has fewer calibrations overall in comparison to Bremer et al. (Bremer, Friis, and Bremer, 2004), but it has more for the Gentianales than have been used in any other publication. However, the age estimates derived from this dataset were much older than those from other works, and should be viewed with caution. The estimated date of divergence for the Gentianales, 143 mya with a total range of 111-175 my, pushed the event back to the nascent years of the Cretaceous or even into the late Jurassic. More than 10 million MCMC generations were pooled, yet the desired estimated sample sizes were never reached, this resulted in a large standard error (Fig. 1.3). The dates presented in this study would negate the invocation of long-distance dispersal for many groups; however, the abandonment of these hypotheses is not recommended. See Table 1.3 for a summary of estimated ages from this study and others.

This is the first publication to combine four chloroplast datasets, parts of which have been presented previously but with narrower sampling. This resulted in a phylogenetic tree for the Gentianales that is fully resolved between families with high support for most basal branches. This will be an asset to the current endeavor to understand the evolution of asterids and to date the divergence of the Gentianales opening up the opportunity to develop more precise biogeographic hypotheses.

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Table 1.1. An inventory of all genera and genes (*matK* , *ndhF* , *rbcL* , and *trnL*) used in the analysis, including information on their orders and infrafamilial classifications.

Order	Family	Infrafamilial Classification	Genus	matK	ndhF	rbcL	trnL
Gentianales	Apocynaceae	Apocynoideae, Apocyneae	*Apocynum*	x		x	x
		Apocynoideae, Wrighteae	*Nerium*	x	x	x	x
		Apocynoideae, Wrighteae	*Wrightia*	x	x	x	x
		Asclepiadoideae, Asclepiadeae	*Araujia*	x	x	x	x
		Periplocoideae	*Periploca*	x	x	x	x
		Rauvolifoideae, Alstonieae	*Alstonia*	x	x	x	x
		Rauvolifoideae, Plumerieae	*Thevetia*	x	x	x	x
		Rauvolifoideae, Vinceae	*Kopsia*	x	x	x	
		Secamonoideae	*Secamone*	x		x	x
	Gelsemiaceae		*Gelsemium*	x	x	x	x
			Mostuea	x	x	x	x
	Gentianaceae	Chironieae, Chironiinae	*Blackstonia*	x		x	x
		Chironieae, Coutoubeinae	*Schultesiana*			x	x
		Exaceae	*Exacum*	x	x	x	x
		Gentianeae, Gentianinae	*Gentiana*	x	x	x	x
		Gentianeae, Swertiinae	*Swertia*	x		x	x
		Helieae	*Irlbachia*			x	x
		Helieae	*Macrocarpaea*			x	x
		Potalieae, Lisianthiinae	*Lisianthius*			x	x
		Potalieae, Potaliinae	*Anthocleista*	x	x	x	x
		Potalieae, Potaliinae	*Fagraea*	x	x	x	x
		Potalieae, Potaliinae	*Potalia*	x	x	x	x
		Saccifolieae	*Curtia*			x	x
	Loganiaceae	Antonieae	*Antonia*		x	x	x
		Antonieae	*Bonyunia*	x	x	x	x
		Antonieae	*Usteria*		x	x	x
		Loganieae	*Geniostoma*	x	x	x	x
		Loganieae	*Labordia*	x	x	x	x
		Loganieae	*Logania*	x	x	x	x
		Loganieae	*Mitrasacme*		x	x	x
		Loganieae	*Mitreola*		x	x	x
		Spigelieae	*Spigelia*	x	x	x	x
		Strychneae	*Gardneria*	x	x	x	x
		Strychneae	*Neuburgia*	x	x	x	x
		Strychneae	*Strychnos*	x	x	x	x
	Rubiaceae	Cinchonoideae, Cephalantheae	*Cephalanthus*	x	x	x	x
		Cinchonoideae, Chicocceae	*Chiococca*	x	x	x	x
		Cinchonoideae, Chiococceae	*Erithalis*		x	x	x
		Cinchonoideae, Cinchoneae	*Cinchona*	x	x	x	x
		Cinchonoideae, Guettardeae	*Guettarda*	x	x	x	x
		Cinchonoideae, Rondeletieae	*Rogiera*	x	x	x	x
		Cinchonoideae, Rondeletieae	*Rondeletia*		x	x	x
		Ixoroideae, Condamineae	*Pinckneya*		x	x	x
		Ixoroideae, Gardenieae	*Gardenia*	x	x	x	x
		Ixoroideae, Ixoreae	*Ixora*	x	x	x	x
		Ixoroideae, Mussaendeae	*Mussaenda*	x	x	x	x

Order	Family	Infrafamilial Classification	Genus	matK	ndhF	rbcL	trnL
	Rubiaceae	Ixoroideae, Vanguerieae	*Vangueria*	x	x	x	x
		Rubioideae, Hedyotideae	*Pentas*	x	x	x	
		Rubioideae, Ophiorrhizae	*Ophiorrhiza*	x	x	x	x
Cornales	Hydrangeaceae		*Hydrangea*	x	x	x	x
Dipsacales	Adoxaceae		*Viburnum*	x	x	x	
	Valerianaceae		*Valeriana*	x	x	x	x
Lamiales	Oleaceae		*Jasminum*	x	x	x	x
	Oleaceae		*Olea*	x	x	x	x
	Plantaginaceae		*Antirrhinum*	x	x	x	x
	Verbenaceae		*Verbena*	x	x	x	x
Solanales	Solanaceae		*Nicotiana*		x	x	x
	Solanaceae		*Petunia*	x	x	x	x

Table 1.2. Sequences of primers used for the *matK*, *ndhF*, *rbcL*, and *trnL* regions in this study and the references for the thermocycling profiles.

Region	Primer name	Sequence	Thermocycling profile
***mat*K**	1198F	CTGTGTTAGATATACGAATACC	Shaw et al,. 2005
	1581R	CTTGATACCTAACATATTGCAT	Shaw et al,. 2005
	1729F	AAGGGTCTATATAAAGCAATT	Shaw et al,. 2005
	2053R	TTAGCRCAAGAYAGTCGAAGTA	Shaw et al,. 2005
	tmK 3914F	GGGGTTGCTAACTCAACGG	Shaw et al,. 2005
	matK -8F	AATTTCAAATGGAAGAAATC	Shaw et al,. 2005
	matK 174F	TGTGAAACGTTTAATTAATC	Shaw et al,. 2005
	matK 174R	CGAKTAATTAAMCGTTTCAC	Shaw et al,. 2005
	matK 503F	TCGCTATTGGGTAAAAGATGC	Shaw et al,. 2005
	matK 503R	GCATCTTTTACCCAATAGCG	Shaw et al,. 2005
	matK 681F	GTGAATACGAATCYATTTC	Shaw et al,. 2005
	matK 900F	TGGAAATTTTACCTTGTCAA	Shaw et al,. 2005
	matK 1309F	GACTTTCTTGTGCTAGAACT	Shaw et al,. 2005
	matk 1628R	CATGCTACATCAACATTTCAG	Shaw et al,. 2005
	tmK -2R	AACTAGTCGGATGGAGTAG	Shaw et al,. 2005
***ndh*F**	NDHF-1F	AGGTAAGATCCGGTGAATCGGAAAC	Kim and Jansen, 1995
	NDHF-2F	AGGTACACTTTCTCTTTGCGGTATTCC	Kim and Jansen, 1995
	NDHF-1R	ATAGATCCGACACATATAAAATGCGGTT	Kim and Jansen, 1995
	NDHF-2R	ACCAAGTTCAATGTTAGCGAGATTAGTC	Kim and Jansen, 1995
***rbc*L**	RBCL-F	ATGTCACCACAAACAGAGACT	Olmstead et al. 1992
	RBCL-R	CTTTTAGTAAAAGATTGGGCCGAG	Olmstead et al. 1992
***trn*L**	7ny54-tmTL-f	CATTACAAATGCGATGCTCT	von Hagen & Kadereit, 2002
	8ny55-tmLF-F	CGAAATCGGTAGACGCTACG	von Hagen & Kadereit, 2002
	9ny56-tmLi-R	GGGGATAGAGGGACTTGAAC	von Hagen & Kadereit, 2002
	10ny57-tmLF-R	ATTTGAACTGGTGACACGAG	von Hagen & Kadereit, 2002
	11ny66-tmLFsp-F	GGTTCAAGTCCCTCTATCCC	von Hagen & Kadereit, 2002

Figure 1.1. Bayesian trees from analyses of individual genes. Posterior probabilities are on branches. A. *matK*. B. *ndhF*. C. *rbcL*. D. *trnL*.

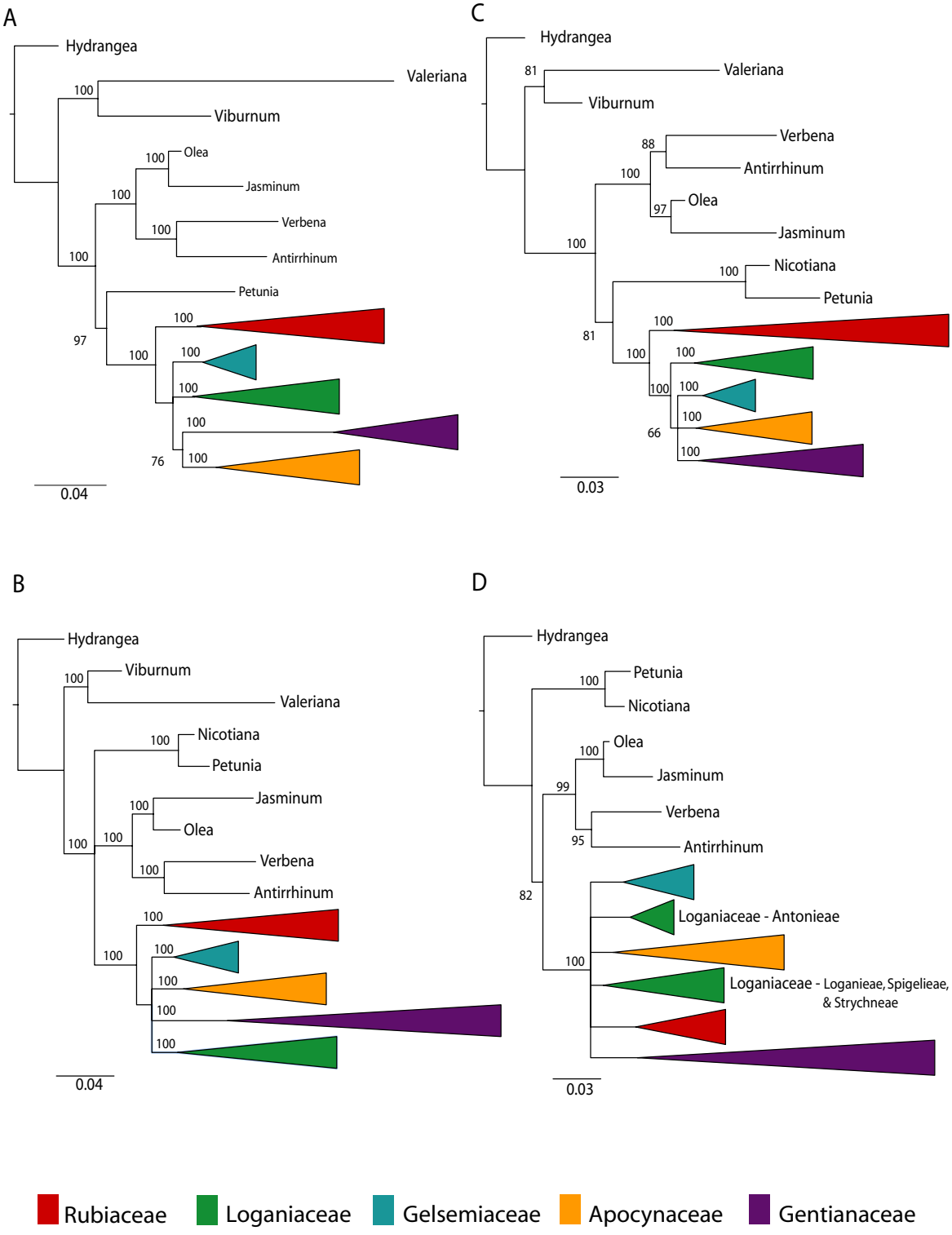


Figure 1.2. Bayesian tree from the results of the combined data set, *matK*, *ndhF*, *rbcL*, and *trnL* region. Posterior probabilities are below the branches.

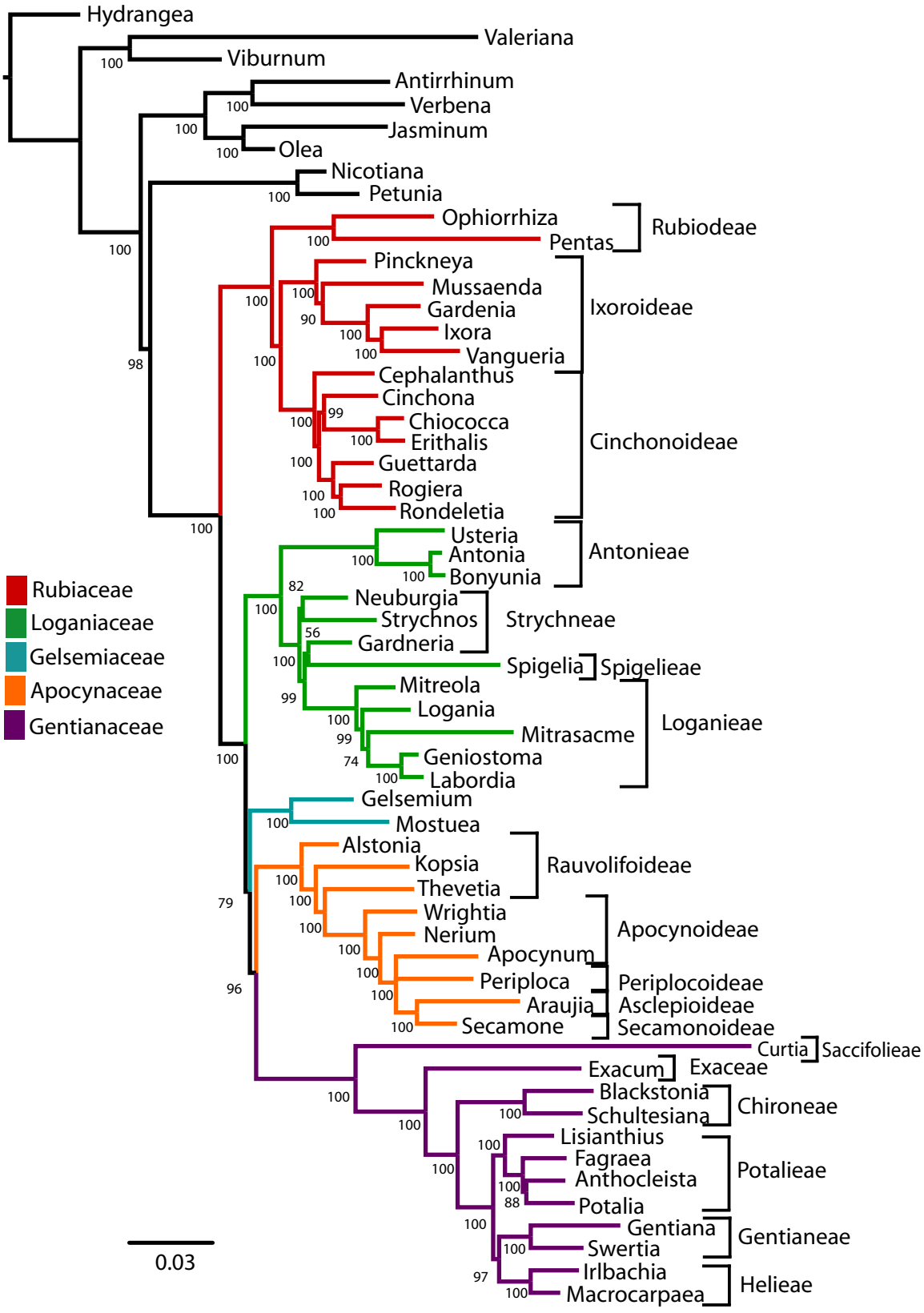


Table 1.3. Summary of estimated ages (my) of the Gentianales from this study and previous works, including the methods used and the number of fossil calibration points. Upper and minimum 95% confidence intervals in parentheses when given.

Method	Gentianales	Apocynaceae	Gelsemiaceae	Gentianaceae	Loganiaceae	Rubiaceae
Fossil-based minimum ages[1]	53	NA	NA	NA	NA	NA
NPRS[2] 1 fossil calibration point	83-89	45-53	NA	NA	NA	61-64
NPRS, MBL, and molecular clock[3] 1 fossil calibration point	60	NA	NA	NA	NA	NA
NPRS, and molecular clock[4] 6 fossil calibration points	108	NA	NA	NA	NA	NA
Bayesian estimation, relaxed clock[5] 4 fossil calibration points	(111) 143 (175)	(54) 69 (84)	(16) 44 (81)	(67.5) 88 (107)	(55) 81 (129)	(70) 105 (130)

[1] Magallón et al. 1999

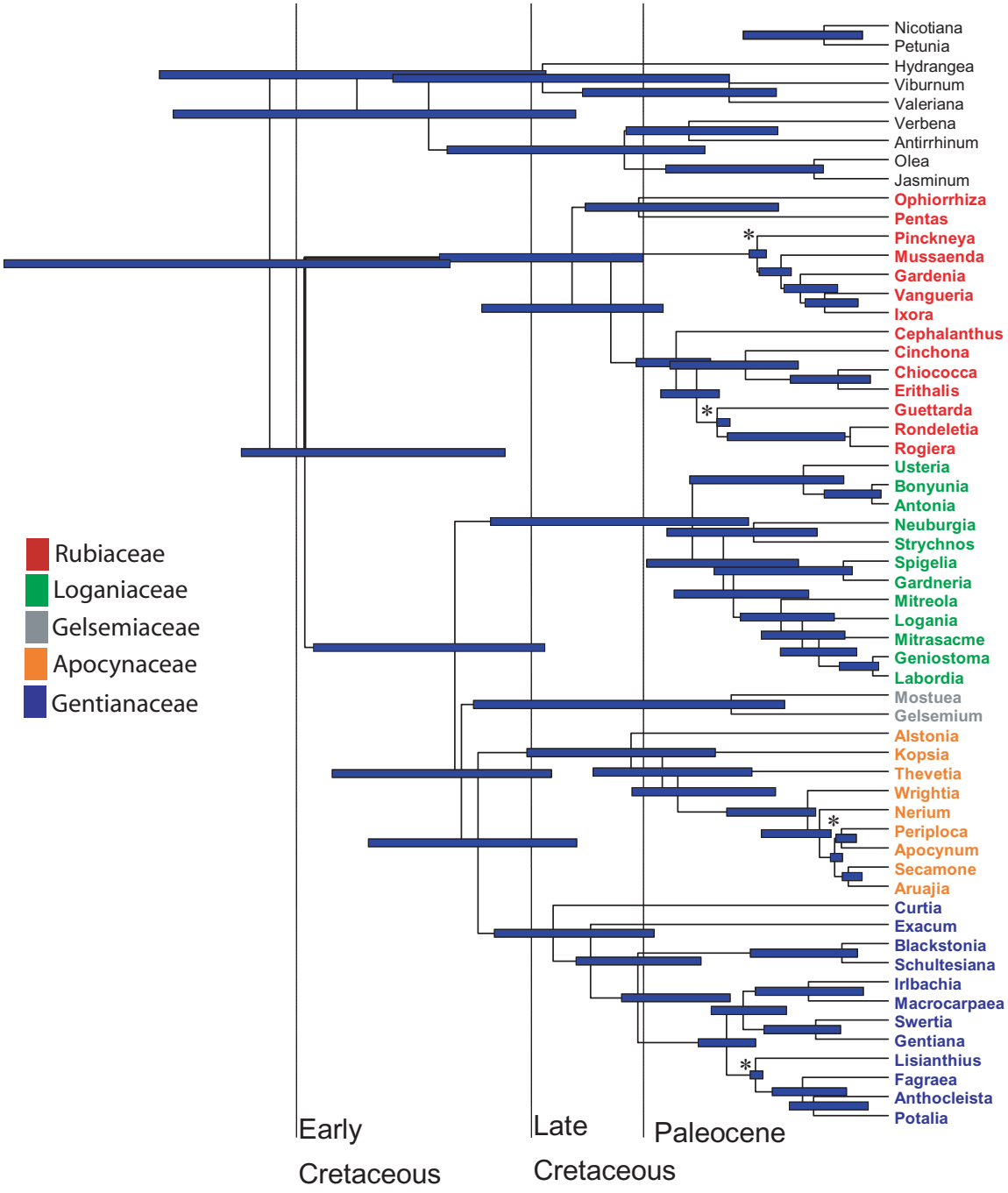
[2] Wikström et al. 2001

[3] Yuan et al. 2003

[4] Bremer et al. 2004

[5] Present study

Figure 1.3. BEAST estimate of phylogeny and divergence dates with error bars. Fossil calibration points marked with an asterisk.



CHAPTER 2

Phylogenetics of Loganiaceae (Gentianales)

Abstract

The angiosperm family Loganiaceae (Gentianales) includes 15 genera classified into four tribes. The phylogenetic relationships within Loganiaceae were evaluated using the nuclear internal transcribed spacer (ITS) region from 13 genera and the *rps*16 chloroplast region with 11 genera. A morphological analysis was conducted to accommodate all genera in the Loganiaceae in this study, including those for which no sequence data was obtained. The secondary structure of the ITS region was estimated for the Loganiaceae, and this information was incorporated into a Bayesian analysis. Maximum parsimony analyses were also performed. Of the four tribes, Antonieae, Loganieae, and Spigelieae were supported as monophyletic in both molecular analyses, and Strychneae appeared as paraphyletic. A combined dataset that includes the ITS, *rps*16, and morphological data yielded a tree that supports the present tribal classification of Loganiaceae. This is the first study using molecular and morphological data that supports the monophyly of Strychneae. A critical examination of the possibility of having delineated Strychneae based on shared morphological characters that are the result of convergence instead of synapomorphies is recommended.

Introduction

Loganiaceae R Br. is a mostly pantropical family of just over 400 species in 15 genera, with some species extending into temperate Australia and North America

(Dunlop, 1996; Struwe et al., In press). Members of the family are mostly woody genera growing as trees and lianas, but also include a few herbs. The most prominent member of Loganiaceae is *Strychnos* L., which has approximately 200 species and a rich cultural history of traditional therapeutic uses.

The family was first suggested by Robert Brown (1814) and validly published by von Martius (1827). The family has undergone numerous revisions that have expanded and contracted its circumscription, ranging from one genus at its smallest (Taktajhan, 1997) to 29 at its largest (Leeuwenberg et al., 1980). The current infrafamilial classification contains four tribes: Antonieae Endl., Loganieae Endl., Spigelieae Dum. (monotypic), and Strychneae Dum. (Struwe and Motley, in press). The tribes Loganieae and Antonieae are supported by molecular data, but Strychneae is not (Backlund et al., 2000). Strychneae includes *Strychnos* and two Asian genera, *Gardneria* Wall. and *Neuburgia* Blume. *Spigelia*, which is restricted to the western hemisphere, was included in the same clade as Strychneae in Backlund et al.'s (2000) study using *ndh*F and *rbc*L sequences, causing the latter to be paraphyletic.

There are few molecular phylogenetic studies that target Loganiaceae (Backlund et al., 2000), and there is a handful that include a scanty sample of loganiaceous taxa as outgroups (Civeyrel et al., 2001; Rova et al., 2002), but there is no publication that treats all Loganiaceae genera in a phylogenetic context. The genes that have been used are from the chloroplast genome and do not support the present tribal classification of the family. Those genera that are frequently neglected are *Phyllangium* Dunlop, *Schizacme* Dunlop, and *Norrisia*. *Phyllanngium* and *Schizacme* were recently segregated from *Mitrasacme* Labill. (Dunlop, 1996), but have received little attention

since their inception. *Norrisia* is a small genus of two species and has never been included in a molecular analysis. A thorough phylogenetic study of the family using a gene from a different cellular compartment would be a useful addition to the current knowledge of Loganiaceae systematics.

Objectives. The goals of this study were threefold. The first goal was to clarify the relationships of genera within Loganiaceae using a quickly evolving gene from the both the chloroplast (rps16) and nuclear (internal transcribed spacer [ITS]) compartments. The second goal was to develop a consensus secondary structure of ITS region for the family as an alignment aid to improve accuracy in phylogenetic reconstruction. The last goal was to perform a morphological analysis in an effort to accommodate *Phyllangium* and *Schizacme* which were unavailable for DNA extraction.

ITS secondary structure. The ITS region of the nrDNA has been used for inferring phylogenetic relationships from the family level to the species level (Baldwin et al., 1995). There are layers of information that can be extracted from this gene. The variability of the primary structure is beneficial to phylogenetic studies, but the ITS region is prone to more indels than coding sequences requiring the insertion of gaps to maintain positional homology, which is critical for phylogenetic studies (Baldwin et al., 1995). As the sampling of a group of species expands, it can become difficult to align ITS sequences between species that are evolutionarily more distant. Goertzen et al. (2003) stressed the value of dense sampling to enhance the alignment process. A broad and dense dataset allows a better understanding of the variability of the gene region and offers the advantage of using intermediary sequences to help with alignment.

Another layer of information that can be extracted from the ITS region is in the secondary structure. Although the primary structure of the ITS region may vary between species, the secondary structure is conserved across different evolutionary lineages. For example, Mai et al. (1997) found similarities in the structure of ITS2 between algal and flowering plants, and Goertzen et al. (2003) developed a minimal secondary structure model of the entire ITS region conserved among members of the Asteraceae. Using the secondary structure as an aid can help ensure positional homology when aligning nucleotides (Kjer, 1995; Hershkovitz et al., 1996; Gottschling et al., 2001; Coleman, 2007). It has also been shown that consideration of the secondary structure in the phylogenetic analysis has improved likelihood-based results (Telford et al., 2005).

It is expected that Loganiaceae's secondary structure will follow certain patterns that were found in other members of the asterids, such as the Asteraceae, Gentianaceae, and the Boraginales (Gottschling et al., 2001; Goertzen et al., 2003; Molina et al., Accepted pending revision). The ITS1 regions of the aforementioned taxa have three to four stems (Goertzen et al., 2003; Molina et al., Accepted pending revision). The blueprint common to the vast majority of eukaryotes for the ITS2 region has four stems (Coleman, 2007).

Materials and Methods

Taxon sampling. Internal transcribed spacer sequences were obtained from a total of 21 Loganiaceous genera. *Geniostoma rupestre* J.R.Forst. & G.Forst. (DQ499095, DQ499096), *Logania albiflora* (Andrews) Druce (DQ358879), *Mitreola*

petiolata (Gmel.) Torr. & A. Gray (AF054635) and all *Spigelia* (AF177992, AF178008, AF178000, AF177991, AF178006) sequences were downloaded from GenBank. Eleven Loganiaceae rps16 sequences were used; *Usteria* and *Mitreola* were not included. Antonieae was used to root the trees as this tribe was placed sister to the rest of the Loganiaceae using *mat*K, *ndh*F, *and rbc*L sequences (Backlund et al., 2000). See Appendix 3 for voucher information.

Morphological data were garnered from the literature and supplemented with observations from herbarium collections. The matrix developed by Struwe and Albert (1997) was used as a starting point and missing Loganaiceae genera were added to it. Some characters were added while others were deleted based on their homoplastic behavior. See Appendix 4 for a description of characters and their states.

DNA extraction, amplification, and sequencing. DNA was extracted from fresh leaf material dried in silica gel or from herbarium specimens. The Qiagen DNEasy Plant Mini Kit was used according to the manufacturer's protocol with the following exception: leaf material was pulverized in a FastPrep machine (Bio 101) for 20 seconds on speed 4 before extraction.

PCR amplification of ITS was performed in 25 μl reaction mixtures using the primers and protocol from Frasier et al. (2008). Amplification of the rps16 region was done using the primers and thermocycling profile from Shaw (2005). PCR reactions were also done using Taq ReadyMix (Sigma) according to the manufacturer's recommended concentration. Sequencing of strands in both directions was done using the same primers as for amplification according to the protocol described in Frasier et

al. (2008) at the Biotech Center, Rutgers University, on an Applied Biosystems GeneAmp 9700 or performed by GeneWiz (Plainfield, NJ).

Sequence alignment. Chromatograms were visualized and edited using Sequencher v. 4.1.4 to v. 4.7 (GeneCodes). Alignments were assembled using MAFFT (Katoh et al., 2002), and adjusted by eye in areas where algorithms tend to falter such as large gaps and repetitive strings of nucleotides. The MAFFT alignment for the ITS dataset was then submitted to RNAsalsa (Misof et al., Submitted) as a prealignment with a constraint file based on the *Strychnos nux-vomica* L. secondary structure. The secondary structure was predicted by RNAstructure v. 4.4 (Mathews et al. 1996-2006). RNAstructure was run using the default settings which produced up to 20 structures. The first structure, which had the lowest free energy, was used for comparison between genera. Stems that were supported by more than a 70% probability as calculated by RNAstructure were included in the constraint file. It was not necessary to work with the secondary structure of the 5.8S region as this part of the sequences were well-conserved and the time spent on secondary structure elucidation would not be useful in the phylogenetic estimation of relationships within Loganiaceae. A diagrammatic depiction of the ITS secondary structure was created using XRNA (B. Weiser and H. Noller, University of California, Santa Cruz) and edited in Adobe Illustrator 10.

Phylogenetic analyses. Maximum parsimony (MP) analyses were conducted using a heuristic search in PAUP*4.0b10 (Swofford, 2002) with all characters of equal weight. One thousand random addition search replicates were run with multrees on, using TBR, and a maximum of 1,000,000 rearrangements per replicate. Jackknife branch support values were calculated in PAUP using 200 replicates with 1000 addition

sequence replicates each. A recommended 36.79% of the data were removed per jackknife replicate (Farris et al., 1996). MrBayes 3.1.2 (Huelsenbeck et al., 2001) was used to perform a Bayesian analysis on the same datasets that used in the MP analyses. For the ITS analysis, the data were divided into two partitions, stems and loops. The doublet model was applied to the stems partition. Both the stems and loops were subjected to the GTR+I+G model as this was selected by the Akaike Information Criterion via MrModeltest v2 (Nylander, 2004). The GTR+I model was selected for the rps16 data. The morphology matrix was modeled using the "standard" model in MrBayes, which is nearly the Jukes-Cantor model (Ronquist et al., 2005).

Two MrBayes runs of 1.2 million generations were performed with four chains each set at the default temperatures. In the combined analysis there were separate partitions for each dataset. Their parameters were unlinked and each was subjected to their own models.

Results

A single fragment was produced from all ITS PCR reactions, which varied in length from 686 bp to 723 bp. The sequences were slightly GC rich, 57%, but the Chi-square test as performed in PAUP did not indicate that there were any significant differences across all sampled individuals. The portions of the SSU and LSU flanking the ITS regions were excluded from the phylogenetic analyses. The alignment had seven ambiguously aligned regions totaling 57 nucleotides that were excluded from the analysis. The final alignment had 677 characters of which 323 were parsimony informative excluding ambiguous areas. The MP analysis yielded one tree that was

678 steps long (CI=0.75, RI=0.82). The Bayesian and MP trees are mostly in agreement, and details will be discussed throughout this section.

The rps16 region was approximately 860 bp long in Loganiaceae. It was difficult to obtain the first half of the region for *Norrisia* and *Mitrasacme*, therefore the second half of the gene region was used for this study. The rps16 matrix had 511 characters and no bases were excluded due to ambiguous alignment. The MP analysis yielded 18 trees that were 155 steps long (CI=0.87, RI=0.82). The Bayesian and MP trees did not disagree, but the MP tree had less resolution.

Secondary structure. The secondary structure of ITS1 in Loganiaceae had four stems: 1A, 1B, 1C, and 1D (Fig. 2.1). The naming convention for the stems was adopted from Goertzen et al. (2003). Stem 1A was missing from *Antonia* Pohl. and *Bonyunia* Rich. Schomb., and was severely abridged in *Norrisia* Gardn. The remaining stems were present in all genera. Helix 1C was identical in all Loganiaceae samples and only one compensatory base change was observed when compared to Asteraceae's 1C. Stems 1A and 1D were the most variable in nucleotide identity of the entire ITS region. Stem 1D was difficult to recognize by eye in Loganiaceae, but it was resolved using RNAstructure.

Many of the features associated with ITS2 in eukaryotes were seen in Loganiaceae's structure. There were four stems: 2A, 2B, 2C, and 2D (Fig. 2.1). Helices 2A and 2D were the most variable of the region, and helix 2B had an expected bulge resulting from a mismatch between pyrimidines (Coleman, 2007). Helix 2C was much longer than the rest and had a relatively conserved feature near the distal end on

its 5' side (Coleman, 2007). Helix 2D was shortened in Spigelieae to include only those bases highlighted in gray in figure 2.1.

ITS. Of the four tribes within the family, Antonieae, Loganieae, and Spigelieae were supported as monophyletic in the MP and Bayesian analyses (Fig. 2.2). Antonieae includes *Antonia*, *Bonyunia*, *Norrisia*, and *Usteria* Willd, but *Usteria* was not included. All of Loganieae's five genera were included in this analysis: *Geniostoma, Labordia* Gaud.*, Logania* R. Br.*, Mitrasacme* Labill. and *Mitreola*. The final monophyletic tribe in this analysis was Spigelieae, which only includes the genus *Spigelia* and was represented by 5 species. Strychneae was not supported as monophyletic in either the MP or Bayesian trees.

The relationships of tribes to one another were contradictory between the two analyses. In the Bayesian tree, Spigelieae was placed, but was in a more basal position in the MP tree. Strychneae was paraphyletic in both trees; it was divided into two subsequently diverging clades in the MP analysis and three subsequently diverging clades in the Bayesian analysis. Conclusions that are supported by both analyses are that Antonieae, Loganieae, and Spigelieae are monotypic tribes, but Strychneae is not.

rps16. Sequences of *Mitreola* and *Usteria* were very similar to one another, but were abnormal in comparison to the rest of the family. They behaved aberrantly in analyses, and when using NCBI's BLAST function were shown to be more closely related to the Asteraceae than the Loganiaceae. Therefore, they were excluded from the analyses.

The MP tree had less resolution than the Bayesian tree (Fig. 2.3). In both trees, Antonieae and Loganieae were monophyletic and Strychneae was not. In the MP tree,

Strychneae was included in a polytomy that also had Spigelieae and Loganieae. In the Bayesian tree, *Neuburgia* is basal to a clade containing the other two Strychneae genera, Spigelieae, and Loganieae. Spigelieae is placed sister to *Strychnos* in a clade that also contains *Gardneria*.

Morphology. The morphology matrix had 59 characters (Tab. 2.1) and the MP analysis resulted in four trees that were 107 steps long (CI=0.62, RI=0.59, Fig. 2.4). The Bayesian tree is in agreement with the MP tree, and varies only in the placement of *Mitreola* and *Mitrasacme*. Antonieae and Strychneae are monophyletic. Loganieae is paraphyletic due to the placement of *Labordia* and *Geniostoma* as sister to Strychneae. The remaining Loganieae genera are distributed throughout a polytomy that also includes Spigelieae and a clade with Strychneae, *Geniostoma*, and *Labordia*.

Combined. The combined data set had 1247 characters, and yielded two most parsimonious trees that were 803 steps long (CI=0.73, RI=0.56). The strict consensus tree had the same topology as the Bayesian tree except for one collapsed branch within Loganiaceae marked on figure 2.5 with an asterisk. All tribes were monophyletic, and three genera have morphology data only (Phyllangium, Schizacme, Usteria). The tree was rooted at Antonieae, and Spigelieae was the next most basal clade, but the support for the branch that separates Spigelieae from Strychneae and Loganieae is weak (0.56 pp). Strychneae and Loganieae were sisters.

Discussion

Secondary structure. The ITS region has been shown to retain information that is useful for conducting phylogenetic analyses targeted at elucidating higher

relationships down through the species level (Baldwin et al., 1995; Coleman, 2007). It can be difficult to align a region with the nucleotide variability of ITS between genera, but the secondary structure provided a guide that was a practical alignment tool.

As predicted, Loganiaceae's secondary structure was similar to other Asterids. It had an additional stem in ITS1, 1D, when compared to Asteraceae (Goertzen et al., 2003), but was nearly identical to Gentianaceae (Molina et al., Accepted pending revision). The presence of A-rich regions immediately following stems 1B, 1C, and 1D were discussed by Gottschling et al. (2001), and observed in Loganiaceae. These regions were useful landmarks when aligning the sequences. Helix 1D was highly variable and difficult to distinguish by manually inspecting the sequences, which highlighted the value of using a modeling program such as RNAstructure (Mathews et al., 2004). This program predicts the secondary structure of a primary sequence via free energy optimization. It has been determined that free energy models have successfully predicted 73% of base pairs although the thermodynamics of these molecules are not completely understood and some RNA sequences can fold into alternative conformations (Mathews et al., 2006).

Trends can be seen in the secondary structure at the tribal level. The absence or truncation of stem 1A in all the sampled members of Antonieae supports the current circumscription of this tribe. Deletions in this area have been recorded earlier as this stem was extremely abbreviated in *Heliotropium*, a member of Boraginales (Diane et al., 2002). The five *Spigelia* species differed from all the other Loganiaceous taxa by having a shortened stem 2D, a possible synapomorphy for Spigelieae and the genus.

The deletions in stems 1A and 2D suggest that these are not under the same selection pressures as the remaining stems.

The differences between the MP and the Bayesian trees could be ascribed to the fundamental differences in the methodologies as well as to the incorporation of the secondary structural information in the Bayesian run. The alignment is the same between the analyses, but the Bayesian analysis takes into account the secondary structure via the doublet model. The topology of the MP and Bayesian trees differed in areas where the branch support values were low, such as the American portion of clade VII. Since these are both heuristic methods, it is not surprising that the approximation of these relationships varied slightly between analyses.

Loganiaceae. The monophyly of Antonieae is a hypothesis that has withstood testing with chloroplast sequence data (Backlund et al., 2000), morphological data (Struwe et al., 1997) and, now, ITS data. This is the first study to include molecular data for *Norrisia*, a small, poorly known Asian genus of two species. A tentative new synapomorphy for this tribe is the absence or truncation of stem 1A in the ITS secondary structure, pending the results of an analysis of *Usteria's* ITS1 sequence.

A review of Loganieae's recent history reveals disparate views on the best way to classify the genera that are currently included in this tribe. In 1980, Leeuwenberg and Leenhouts placed *Geniostoma*, *Labordia*, and *Logania* in tribe Loganieae. In 1994, Struwe et al. whittled down Loganiaceae so that it only included *Logania*, *Mitreola*, and *Mitrasacme* and did not use any tribal classifications. In 1997, (Taktajhan) reduced Loganiaceae to a single genus, *Logania*, so obviously did not have tribes. The most recent classification has *Geniostoma, Labordia, Logania, Mitrasacme,* and *Mitreola* in

Loganieae in Loganiaceae (Struwe et al., In press), a classification that is supported by chloroplast data (Backlund et al., 2000) and our ITS data.

Two genera that are Australian endemics have rather recently been segregated from *Mitrasacme*: *Phyllangium* Dunlop and *Schizacme* Dunlop (Dunlop, 1996). Only morphological data for these genera were included in this study. The results here suggest that *Phyllangium* and *Schizacme* be included in Loganieae, but efforts were not made to test their monophyly.

Spigelieae is a monophyletic group in this analysis; the exclusion of *Mitrasacme* and *Mitreola* from this tribe is supported. Unfortunately, the relationship of Spigelieae to other Loganiaceous genera is ambiguous. In the combined tree, Spigelieae is placed basal to Strychneae and Loganieae, but this has low branch support (Fig. 2.5). There is conflict between the Bayesian and MP analyses in the placement of this tribe. The five *Spigelia* sequences were very similar to one another, and are placed together on a rather long branch (Fig. 2.2). Struwe and Albert (1997) were also unable to place Spigelieae into the framework of the Loganiaceae phylogeny with certainty. More data are necessary to understand Spigelieae's relationship to other Loganiaceous taxa.

Two independent analyses have failed to support the monophyly of Strychneae (Struwe et al., 1997; Backlund et al., 2000). Strychneae was paraphyletic in both molecular analyses conducted here, as well as in additional chloroplast analyses using a combination of *ndh*F, *rbc*L, *mat*K, and *trn*L sequences (Ch. 1). This is the first cladistic phylogenetic study that supports the monophyly of this tribe, but only with the addition of morphological data. Examples of characters that support Strychneae are a woody habit, axile placentation, a fleshy placenta, and the presence of an indehiscent fruit

(Struwe et al., 1997). However, the woody habit is also a feature of Antonieae, *Geniostoma*, and *Labordia*, and Spigelieae and *Logania* can be herbaceous or woody. *Geniostoma* and *Labordia* also have axile placentation and fleshy placentas. The indehiscent fruit is unique to Strychneae, but the fruit type varies from berry to drupe (Leeuwenberg et al., 1980). In addition, the wood anatomy is heterogeneous (Mennega, 1980). The repeated conflict in relationships between *Gardneria, Neuburgia*, and *Strychnos* calls attention to the need for an in-depth study on this group. Careful consideration of the characters that are potential synapomorphies for Strychneae should be undertaken to determine if they are features of a recent common ancestor or examples of convergence.

Conclusions. This work presents the first estimation of the secondary structure for the ITS region for Loganiaceae. Analysis of the ITS and rps16 data provide additional support for the monophyly of tribes Antonieae, Loganieae, and Spigelieae in Loganiaceae, but did not support the monophyly of Strychneae. The inclusion of morphological data with the molecular data in a combined analysis resulted in a tree that supports the current tribal classification of Loganiaceae.

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Figure 2.1. Examples of typical Loganiaceae ITS1 and ITS2 secondary structures as deomnstrated by folding the sequence of *Strychnos nux-vomica*. Stem 1A is missing in tribe Antonieae and stem 2D is abridged in Spigelieae to only those bases in the gray box.

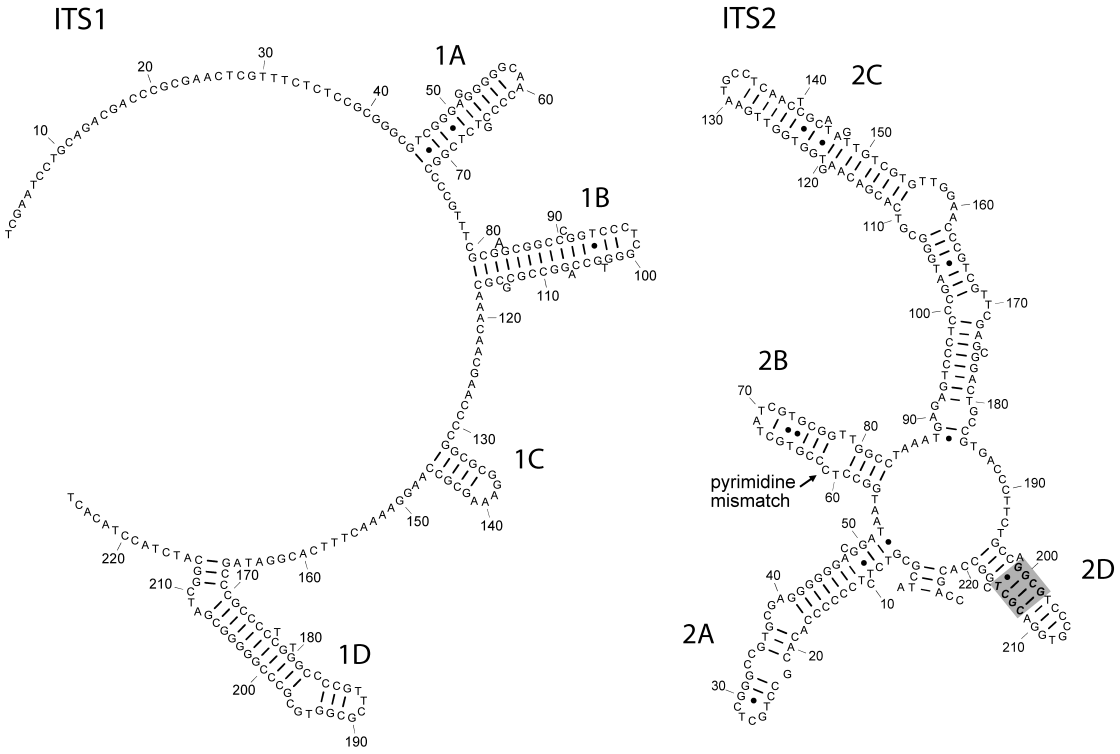


Figure 2.2. ITS tree. A. Bayesian tree with posterior probabilities on branches. B. MP tree with jackknife values on branches.

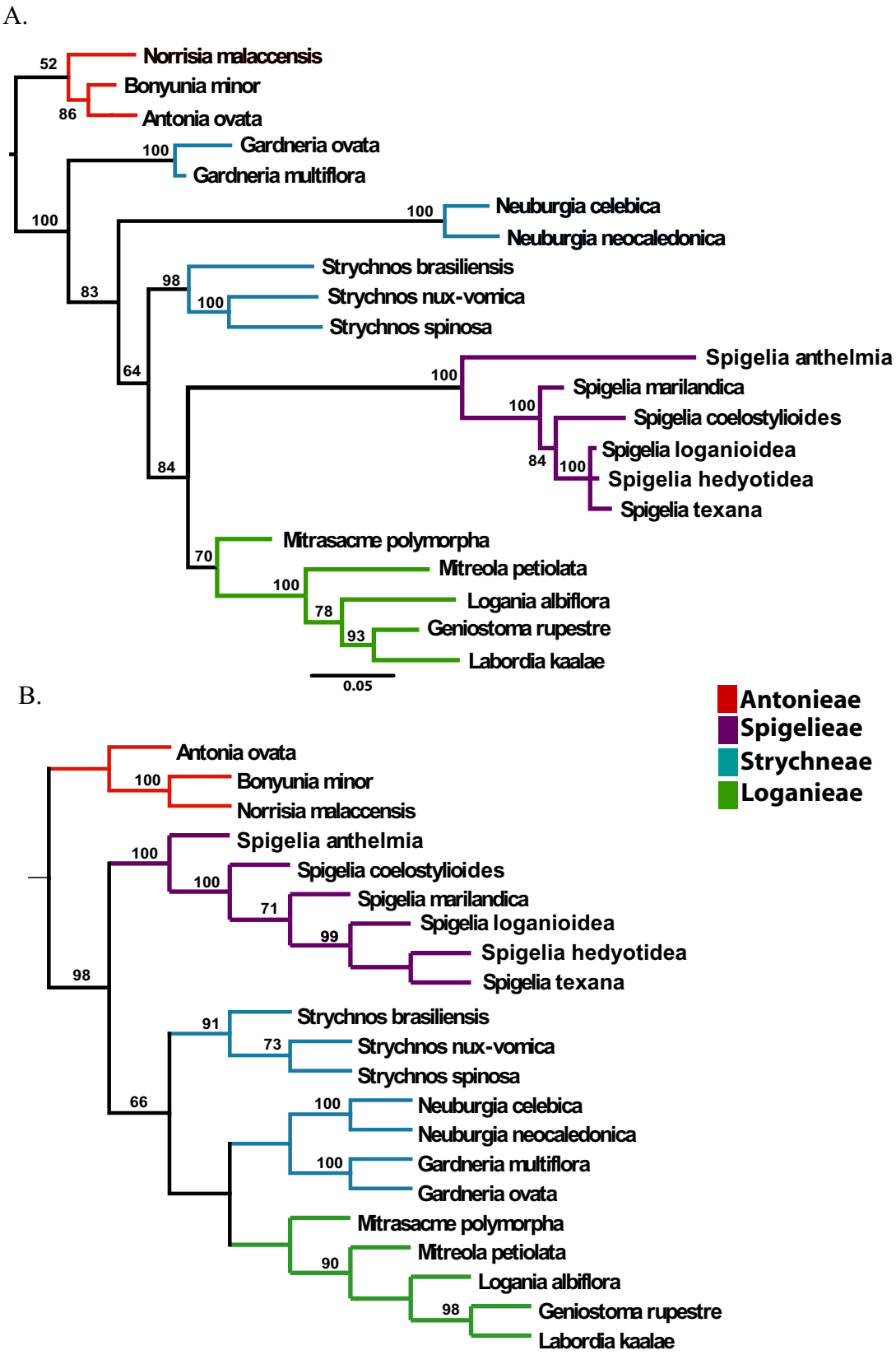
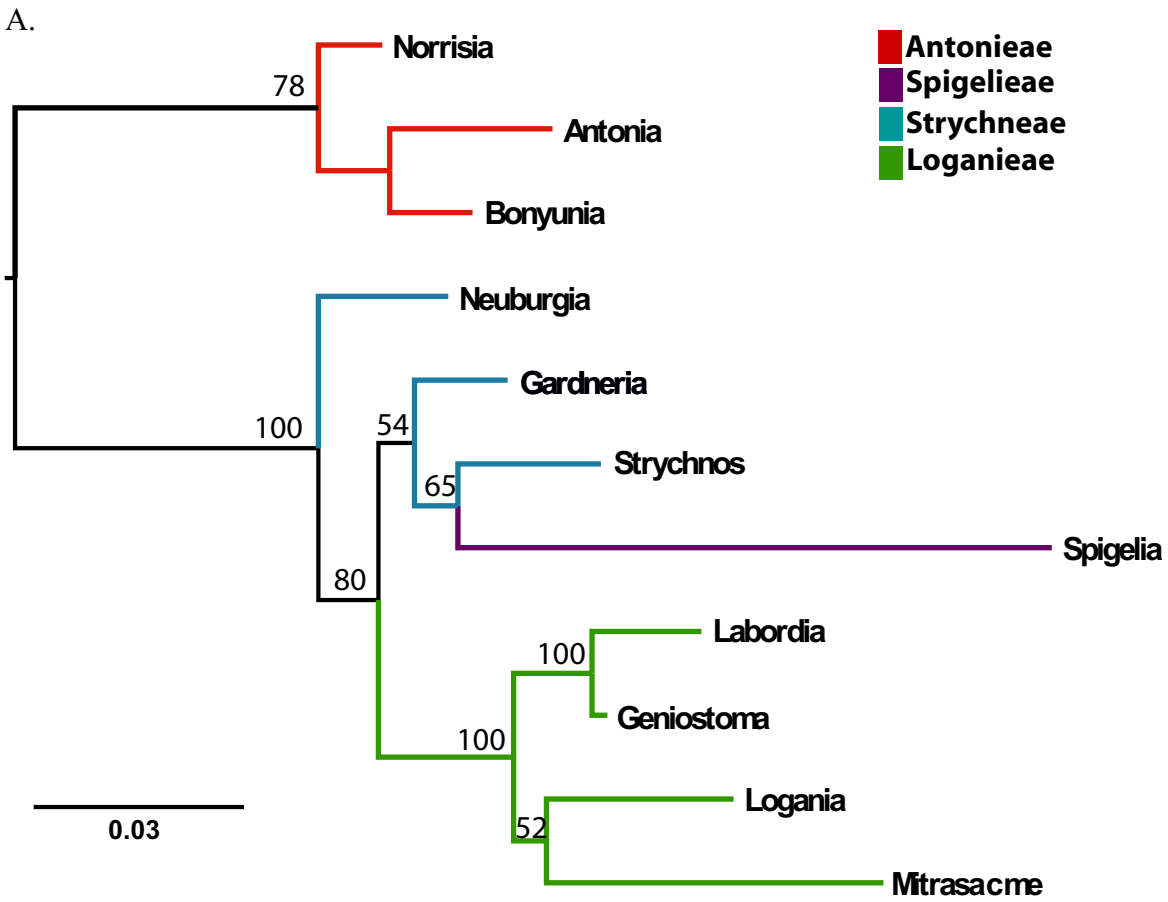


Figure 2.3. *rps16* tree. A. Bayesian tree with posterior probabilities on branches. B. MP tree with jackknife values on branches.



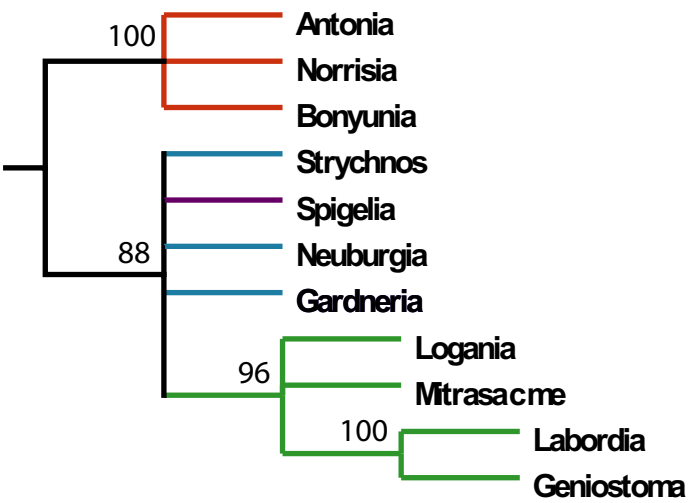


Table 2.1. Loganiaceae morphology matrix of 58 characters.

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Antonia	1	0	0	1	1	0	2	0	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	0	1	0
Bonyunia	1	0	-	0,1	0	0	1	0	0	0	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	1	1	0
Gardneria	1	0	0	?	1	0	1	1	0	0	0,1	0	0	1	1	0	0	0,1	0	0	0	0,1	0	0	1	1	0	0
Geniostoma	1	?	1,2	?	1	0	1	0,1	0	1	0	0	0	0,2	0	0	0	1	0	0	1	0	0,1	0	0	1	?	0
Labordia	1	0	1	?	1	0	1	0	0	1	0	0	1	2	0	0	1	1	0	0	?	0	?	?	0	0	0,1	0
Logania	0,1	-	0,2	-	0,1	0	1	0	0	0,1	0	-	0	2	0,1	0	0,1	1	0	0	0	-	0	-	0	0	0,1	0
Mitrasacme	0	?	2	-	0,1	0	1	0,1	0,2	0	1	1	0	1	0	0	0	1	0	0,1	0	0	0,1	0,1	0	0,1	0	0
Mitreola	0	?	0,1	1	0	0	1	0,1	1	0	0	0	0	2	0	0	0	1	0	0	0	1	0	0	0	1	0	1
Neuburgia	1	0	2	?	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0,1	0	0	1	1	0
Norrisia	1	1	-	?	0	0	2	0	0	0	0	1	0,2	1	0	0	1	1	0	1	0	0	0	1	0	0	1	0
Phyllangium	0	?	2	-	-	0	0	1	2	0	1	-	3	1	-	0	-	-	0	0	-	-	-	-	0	-	0,1	0,1
Schizacme	0	?	2	-	1	0	-	0	2	0	1	-	1	1,2	0	0	0	0	0	0	-	-	-	-	0	1	0	1
Spigelia	0,1	?	1,2	1	1	0	1	0	1	0	0	?	0,1	1	0	1	0	0	0	0,1	0	0	0	0	0	0	1	0
Strychnos	1	0	-	?	0,1	1	1	0,1	0	0	0,1	0	0	1	0	0	0,1	1	0	0	?	0	0	0	0	0	0	0
Usteria	1	0	-	1	0	0	1	0	0	0	1	1	2	1	0	1	1	1	1	1	0	0	0	0	?	0	0	0

Taxon	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
Antonia	0	1	0	1	0	1	1	0	0	1	2	1	0	0	0	1	0	1	0	1	0	0	0	1	1	1	0	0
Bonyunia	1	1	0	1	0	1	1	0	0	1	2	1	-	-	0	1	0	0	1	1	0	0	0	1	1	1	0	0
Gardneria	0	0	0	0	0	0	0	1	0	0,3	1	0	2	-	0	0	0	0	?	0	0	0	0	1	0	0	?	1
Geniostoma	0	0	0	0	0	0	1	0	2	3	1	0	1	0	1	0	0	0	1	0	1	0	1	1	0	0	?	?
Labordia	0	0	0	0	0	0	1	0	2	1	0	0	0	0	1	0	?	0	1	0	1	0	1	1	0	0	?	?
Logania	0	-	1	0	0	1	1	0	0	1,3	0	0	1	0	0	0	0	-	0	0	0	1	0	1	0	0	?	-
Mitrasacme	0	1	1	0	0,1	1	1	0	0	1	0	0	0	-	0	0,1	0,1	-	-	0	0	0	0	1	0	0	?	-
Mitreola	0	1	1	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	-	0	0	0	0	1	0	0	?	-
Neuburgia	0	0	0	0,1	0	0	1	1	0	0,2	1	0	-	-	0	0	0	0	0	0	0	0	0	0	0	0	?	?
Norrisia	0	1	0	-	0	1	1	0	0	1	0	0	-	-	0	?	0	1	0	1	0	0	0	1	1	1	0	0
Phyllangium	-	-	1	0	1	1	1	0	0	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Schizacme	0	1	1	0	0	1	1	0	0	1	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spigelia	1	1	0	0	0,1	1	1	3	1	1	0	0	0,2	-	0	0	1	0	0	0	0	0	0	0	0	0	?	0
Strychnos	0	0	0	-	0	0	0	1	0	0	1	0	-	-	0	0	0	0	1	0	0	0	0	1	0	1	1	1
Usteria	0	1	0	-	0	1	1	0	0	1	2	1	-	-	0	?	0	1	1	1	0	1	0	1	0	0	?	?

Taxon	57	58
Antonia	0	?
Bonyunia	0	?
Gardneria	1	?
Geniostoma	-	0
Labordia	-	0
Logania	0	2
Mitrasacme	-	?
Mitreola	-	0
Neuburgia	-	?
Norrisia	0	?
Phyllangium	-	?
Schizacme	-	?
Spigelia	0	2
Strychnos	1	1
Usteria	-	1

Figure 2.4. Trees resulting from the analysis of the morphological data. A. Bayesian tree with posterior probabilities on branches. B. MP tree with jackknife values on branches.

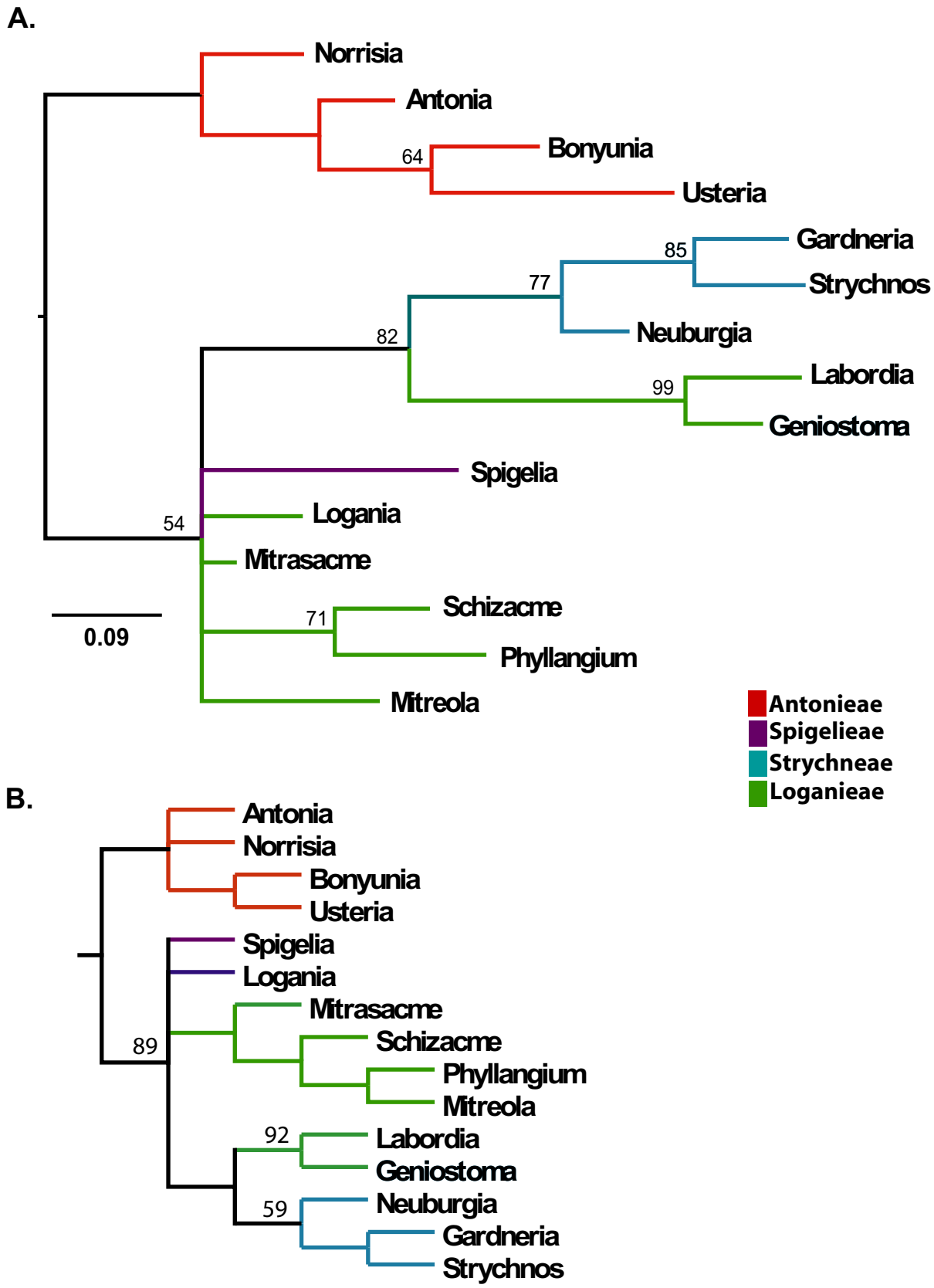
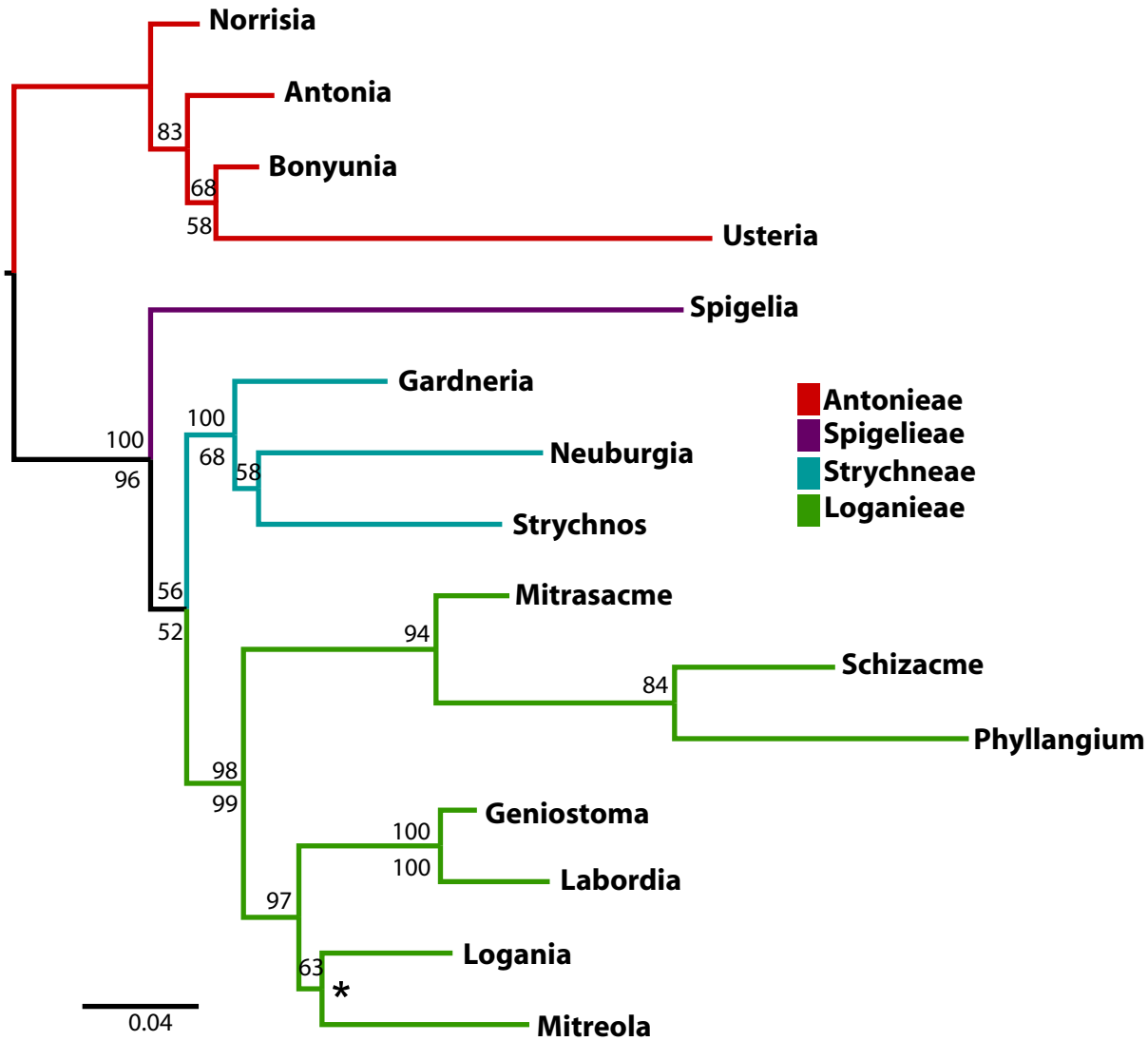


Figure 2.5. The Bayesian and MP analyses of ITS, *rps16*, and morphology data yielded nearly the same topology. Numbers above the branches are posterior probabilities, numbers below the branches are jackknife values. The branch marked with an asterisk collapsed in the strict consensus MP tree.



CHAPTER 3

Phylogeny, Biogeography, and Sectional Classification of Pantropical *Strychnos* (Loganiaceae: Gentianales)

Abstract

Strychnos is the largest genus in Loganiaceae (Gentianales) with approximately 200 species distributed throughout the tropics. The nuclear internal transcribed spacer (ITS) region from 102 accepted species were obtained and analyzed via Bayesian and maximum parsimony methodologies to assess the phylogenetic relationships between *Strychnos* species. *Strychnos* is classified into 12 sections, of which one was supported as monophyletic, sect. *Spinosae*. The resurrection of section *Longiflorae* is recommended and a new section including the *Strychnos jobertiana* group is recommended. Preliminary biogeographic results indicate large groups restricted to whole continents, and with a basal split between a Latin American and an African clade. Within the African clade, there was infrequent and later dispersal to Madagascar, the Neotropics, tropical Asia, and northernmost Australia.

Introduction

Strychnos L. is the largest genus in Loganiaceae with approximately 200 species that grow in tropical rainforests and savannas as lianas, shrubs, or small trees. In the neotropics *Strychnos* is distributed from Mexico down through Bolivia. In the paleotropics it is found throughout tropical Africa and Madagascar, in India, Sri Lanka,

southeast Asia and the northern tropical part of Australia (Krukoff et al., 1942; Bisset et al., 1973; Leeuwenberg et al., 1980).

Strychnos is probably most famous in popular science and culture for its production of the toxin strychnine, which is commercially extracted from *S. nux-vomica* (Samuelsson, 1992), a species from southeast Asia (from India to Vietnam and into tropical China (Bisset et al., 1973)). However, there is a rich cultural history associated with many species of this genus.

More than 12 American species of *Strychnos,* such as *S. toxifera,* are used as primary or secondary ingredients in the dart poison, curare (Krukoff et al., 1942; Krukoff et al., 1969; van Andel, 2000). In regions of Africa certain *Strychnos* species, such as *S. aculeata*, are used as fish poisons and for treating parasitic infections like guinea worm (Burkill et al., 1995). In Madagascar, *Strychnos myrtoides* has been combined with more conventional drugs to treat malaria (Rafatro et al., 2000), and in India, *S. potatorum* seeds are used to settle turbid water (Cooke, 1871; Gupta et al., 1992). This is just a sample of the many ethnobotanical applications associated with *Strychnos*.

The various therapeutic and other ethnobotanical uses for *Strychnos* have made the genus an attractive subject to many chemical investigators (Marini-Bettòlo et al., 1967; Bisset et al., 1971a; Yan et al., 2006). Multiple in-depth taxonomic studies have also been conducted on the genus (Krukoff et al., 1942; Leeuwenberg, 1969; Krukoff, 1972; Krukoff, 1979; Leeuwenberg et al., 1980). Although there is a sizeable body of scientific literature available for *Strychnos* in the areas mentioned above, this is the first phylogenetic and biogeographic study ever published.

Objectives. The goals of this are to ascertain the monophyly of *Strychnos* and to evaluate the infrageneric classification and biogeography from a phylogenetic perspective for the first time.

Generic classification. *Strychnos* has been divided into 12 sections based on morphological and anatomical characters in the most recent sectional treatment of the genus by Leeuwenberg and Leenhouts (1980). At the time of Leeuwenberg and Leenhouts' publication, Africa had the greatest number of described *Strychnos* species, and these were classified into 11 of the 12 sections. The only section that does not have a representative in Africa is sect. *Strychnos*, whose species are American or Asian. Of the 12 sections, six occur only in Africa: *Densiflorae* Duvign., *Dolichanthae* Duvign., *Spinosae* Duvign., *Aculeatae* Duvign., *Phaeotrichae* Duvign., and *Scyphostrychnos* (S.Moore) Leeuwenberg. The last three sections are monotypic. The sections with the greatest representation in Africa are *Lanigerae* A.W. Hill and *Breviflorae* Prog. with 12 species each. However, the bulk of *Lanigerae* is Asian (Leeuwenberg et al., 1980), and the bulk of *Breviflorae* is American (Krukoff, 1972). Section *Breviflorae* was divided into two subsections, *Breviflorae* and *Eriospermae* Krukoff & Barneby, by Krukoff and Barneby (1969) in their treatment of the American species, but this was omitted in Leeuwenberg and Leenhout's (1980) classification. Therefore, the African species of this section are currently not assigned to either subsection.

The American species of *Strychnos* are only in three sections, the aforementioned *Breviflorae* and *Strychnos* plus *Rouhamon* (Aubl.) Prog. (Krukoff et al., 1942; Leeuwenberg et al., 1980). The Asian species are divided into four sections: *Strychnos*, *Brevitubae* A.W. Hill, *Lanigerae*, and *Penicillatae* A.W. Hill (Leeuwenberg et al., 1980).

Unlike many of the African sections that are restricted to the African continent, none of the American and Asian sections are unique to their continents. For example, section *Strychnos* is divided between the Americas and Asia, and sections *Brevitubae* and *Penicillatae* are in both Asia and Africa.

The primary purpose of Leeuwenberg and Leenhout's classification was for identification of *Strychnos* species and they utilized mostly gross morphological characters (Leeuwenberg, 1969). The sections group species with a common suite of cardinal characters relying heavily on the ratio of corolla tube length: corolla lobe length as well as the density and location of pubescence on the corollas. However, many of the sections have overlapping character descriptions suggesting that the sections are heterogeneous, and some might be based on symplesiomorphies. For these reasons, the monophyly of many of the sections is in doubt.

A phylogenetic perspective on *Strychnos* will be valuable for studying evolutionary questions on a widespread and ecologically variable group of plants. For those studying the phytochemistry or ecology of *Strychnos* and other loganiaceous genera, this phylogenetic study will be a new tool to enhance experimental design or interpretation of results.

Materials and Methods

Taxon sampling. Internal transcribed spacer sequences were obtained from a total of 128 *Strychnos* individuals primarily from herbarium material (see Appendix 5 for voucher information). They represent 102 of the approximately 200 currently accepted species from all 12 sections. *Strychnos* herbarium material was determined by the

authors. Two outgroups were included, *Gardneria multiflora* and *Neuburgia neocaledonica*, which are also members of tribe Strychneae.

DNA extraction, amplification, and sequencing. DNA was extracted from fresh leaf material dried in silica gel or from herbarium specimens. The Qiagen DNEasy Plant Mini Kit was used according to the manufacturer's protocol with the following exception: leaf material was pulverized in a FastPrep machine (Bio 101) for 20 seconds on speed 4 before extraction.

PCR amplification of ITS was performed in 25 μl reaction mixtures using the primers and protocol from Frasier et al. (2008). A different forward primer for *Strychnos aculeata* was used: GGAAGTAGAAGTCGTAACAAGG; this is a slightly modified version of White et al.'s ITS5 (1990). This primer was employed for *Strychnos aculeata* because amplification attempts of the first half of the ITS region for two different individuals produced a band that was slightly shorter than all the other *Strychnos* species and sequenced poorly. Using White et al.'s ITS5 primer produced a band of the expected length that was sequenced successfully, and, when BLASTed against NCBI's GenBank database, was closely related to the ingroup. PCR reactions were also done using Taq ReadyMix (Sigma) according to the manufacturer's recommended concentration. Sequencing of strands in both directions was done using the same primers as for amplification according to the protocol described in Frasier et al. (2008) at the Biotech Center, Rutgers University, on an Applied Biosystems GeneAmp 9700 or performed by GeneWiz (Plainfield, NJ).

Sequence alignment. Unedited sequences were visualized and edited using Sequencher v. 4.1.4 to v. 4.7 (GeneCodes). A prealignment was assembled using

MAFFT (Katoh et al., 2002) then submitted to RNAsalsa with a constraint file based on the *Strychnos nux-vomica* secondary structure. The secondary structure was predicted by RNAstructure v. 4.4 (Mathews et al. 1996-2006).

Phylogenetic analyses. Maximum parsimony (MP) analyses were conducted using a heuristic search in PAUP*4.0b10 (Swofford, 2002) with all characters of equal weight. One thousand random addition search replicates were run with multrees on, using TBR, and a maximum of 1,000,000 rearrangements per replicate. Jackknife branch support values were calculated in PAUP using 200 replicates with 1000 addition sequence replicates each. A recommended 36.79% of the data were removed per jackknife replicate (Farris et al., 1996). Phylogenetically informative gaps were coded using the complex indel coding method of Simmons and Ochoterena (2000).

MrBayes 3.1.2 (Huelsenbeck et al., 2001) was used to perform a Bayesian analysis on the same dataset that was used in the MP analysis. The data were divided into two partitions: sequence data and phylogenetically informative gaps coded as present or absent (see methodology above). The GTR+I+G model was used as this was selected by the Akaike Information Criterion via MrModeltest v2 (Nylander, 2004). Phylogenetically informative gaps were modeled using the "standard" model in MrBayes, which is nearly the Jukes-Cantor model (Ronquist et al., 2005). Two MrBayes runs of one million generations were performed with four chains set at the default temperatures.

Results

A single fragment was produced from all ITS PCR reactions. The sequences were slightly GC rich, 57%, but the Chi-square test as performed in PAUP did not indicate that

there were any significant differences across all sampled individuals. The portions of the SSU and LSU flanking the ITS regions were excluded from the phylogenetic analyses. The alignment had five ambiguously aligned regions totaling 42 nucleotides that were excluded from the analysis. The final alignment had 677 characters of which 276 were parsimony informative excluding ambiguous areas. The MP analysis yielded 6289 trees that were 1623 steps long (CI=0.40 including uninformative characters, RI=0.73). The Bayesian and MP trees are mostly in agreement, and details will be discussed throughout this section (Figs. 3.1-2).

Strychnos monophyly and sectional divison. *Strychnos* was monophyletic in both analyses. The relationships among *Strychnos* species were similar between the MP and Bayesian analyses of the ITS data (Figs. 3.1-2). The ITS data supported the monophyly of *Strychnos*, and the species were distributed among nine clades indicated with Roman numerals in Figures 3.1 and 3.2. Some general phylogeographic patterns were observed. To assist in interpreting these, the provenance of the samples are indicated by a symbol after the name in Fig. 3.1 (* = Americas, †= Asia, ‡ = Australia, ¤ = Madagascar, and no symbol = Africa).

The earliest diverging clade, clade I, contains *Strychnos brasiliensis* Mart. of sect. *Breviflorae* subsect. *Breviflorae,* which is placed sister to all the sampled members of sect. *Breviflorae* subsect. *Eriospermae*. Subsection *Breviflorae* is polyphyletic in this analysis, but *Eriospermae* is monophyletic. The bulk of sect. *Breviflorae* subsect. *Breviflorae* is included in clade VII. Only the American species in this section were divided into subsections, and the seven sampled African species of sect. *Breviflorae* were spread throughout the tree.

Clade II includes two monotypic sections, *Aculeatae* (*Strychnos aculeata*) and *Scyphostrychnos* (*S. camptoneura*), plus all sampled members of section *Dolichanthae.* *Aculeatae* is sister to *Dolichanthae* + *Scyphostrychnos*. *Dolichanthae* is paraphyletic due to the placement of *Scyphostrychnos*. All individuals in this clade are from west-central Africa.

The placement of clades III-VIII varies between the Bayesian and MP trees (Fig. 3.1 and 3.2, respectively). In the Bayesian tree there is a polytomy combining clades III, IV, V, VI + VII, and VIII + IX. The strict consensus MP tree is more resolved in this portion of the tree, but has low branch support for the placement of these clades. In the MP tree, clades III and V are in a polytomy with clade IX, and clades IV and VI are sisters, which together are placed sister to clade VII.

Clade III is a combination of sections *Rouhamon* and *Breviflorae*, all individuals are from Gabon except for *S. decussata*, this individual was from Madagascar. Clade IV includes four of the six sampled members of section *Brevitubae*. The remaining two *Brevitubae* members, *Strychnos afzelii* and *S. umbellata*, are in clades VII and IX, respectively. Clade V is a group of African taxa from the west-central portion of the continent. This clade has members from sections *Breviflorae* (*S. malchairii*), *Brevitubae* (*S. dale, S. elaeocarpa, S. floribunda*, and S. *usambarensis*), *Penicillatae* (*S. longicaudata*) and *Rouhamon* (*S. cuniculina*). Clade VI contains two species, *S. boonei* (sect. *Rouhamon*) and *S. campicola* (sect. *Breviflorae*), both are from Cameroon.

Clade VII is a larger assemblage than the others. The most basal subclade of this group includes samples of section *Breviflorae* from Gabon (*Strychnos malacaclados* and *S. angolensis*) and South Africa (*S. henningsii*). The next diverging subclade includes *S.*

myrtoides (Tanzania) and *S. mosteuoides* (Madagascar) of section *Penicillatae*. Malagasy species of section *Penicillate* constitute the subsequent branches. The next node is a polytomy that contains species from a wide geographical range, Madagascar (*S. matopensis*), west/central Africa (*S. afzelii*) plus Australia (*S. arborea*), and a large American subclade. There is relatively little resolution among the American species and sections *Strychnos*, *Rouhamon*, and *Breviflorae* subsection *Breviflorae* are combined into the same subclade. Clade VIII has two African species placed sister to a clade with Asian samples and one Australian sample (*S. lucida*). *Strychnos potatorum*, of section *Rouhamon*, was collected from Malawi and *S. icaja*, of section *Breviflorae*, was collected from Cameroon. The Asian and Australian species in this clade are all members of section *Strychnos*.

Clade IX is the second largest group in this tree. The only section that includes more than one species that was supported as monophyletic was the African *Spinosae*, which is a subclade of clade IX. The monotypic section *Phaeotrichae* is placed in clade IX together with six American species belonging to sections *Strychnos* and *Rouhamon*.

Multiple individuals of 16 species were sampled. Nine of those species appear to be monophyletic: *Strychnos aculeata, S. asterantha, S. axillaris, S. lucida, S. minor, S. mosteuoides, S. schultesiana, S. spinosa,* and *S. staudtii.* These species were reduced to one monophyletic terminal in the figures with the number of individuals sampled in parentheses after the name. *Strychnos angustiflora* appeared as paraphyletic and is placed sister to *S. lucida*. The remaining six species with more than one accession showed up as polyphyletic: *S.angolensis, S. barteri, S. darienensis, S. henningsii, S. panamensis,* and *S. tricalysioides*.

Discussion

The monophyly of *Strychnos* was previously questioned when an analysis using morphological and chemical data placed *Gardneria* and *Neuburgia* within the genus (Struwe et al., 1994). In addition, the inclusion of *Scyphostrychnos camptoneura*, the sole member of its genus, into *Strychnos* broadened the morphological variability of a genus that was already quite variable (Leeuwenberg, 1965). The study presented here is the first to concentrate on *Strychnos*, and our results support its monophyly with high posterior probabilities and jackknife values. The sections within *Strychnos*, however, were not supported as monophyletic, with one exception, sect. *Spinosae* (Fig. 3.1, clade IX).

Strychnos sect. *Spinosae* is an African section with four species. There are multiple synapomorphies associated with this group such as the presence of a narrow, white, penicillate-hairy corona and interpetiolar stipules, as well as the absence of interxylary phloem in the wood (Leeuwenberg et al., 1980; Mennega, 1980). Interxylary phloem is a common feature of *Strychnos* and its presence has been recorded for all species whose wood anatomy has been investigated, except for those in sect. *Spinosae* and one other species, *S. henningsii* sect. Breviflorae (Mennega, 1980). Mapping of this character on the MP and Bayesian trees suggests that *S. henningsii*'s loss of interxylary phloem was independent of the loss of this character in sect. *Spinosae*. Chemical data provide additional support for *Spinosae* as this section was one of only two (the other being *Densiflorae*) that is chemically homogenous in that no members produced significant quantities of tertiary alkaloids (Bisset et al., 1971b). The other sections,

excluding the three monotypic ones, had a large variation in the production of tertiary alkaloids (Bisset et al., 1971b).

There are eight species in the African sect. *Densiflorae* of which five were included in this analysis: *Strychnos lucens*, *S. innocua*, *S. madagascariensis*, *S. pungens*, and *S. staudtii*. These species, excluding *S. staudtii*, form a monophyletic group in clade IX with high branch support (Fig. 3.1). The exclusion of *S. staudtii* from sect. *Densiflorae* would recircumscribe the section and maintain its monophyly, and also be supported by morphological data. The *Densiflorae* section is associated with the following characters: a simple ring of "brush-like" lanate hairs inside the corolla throat, a pilose pistil, and yellow or orange fruits (Leeuwenberg et al., 1980). In contrast, *S. staudtii* has two separate rings of hair in the corolla, a glabrous pistil, and a white fruit.

Section *Dolichanthae* is an African group with nine species of which four were sampled. It is paraphyletic due to the inclusion of *S. camptoneura*, the only member of sect. *Scyphostrychnos* (Fig. 3.1, clade II). *Scyphostrychnos* is unique in having narrowly winged seeds (Leeuwenberg et al., 1980), an obviously autapomorphic trait. Another monotypic section, *Aculeatae*, is sister to *Dolichanthae* + *Scyphostrychnos* in this analysis. *Strychnos aculeata* is an African species that differs from all other *Strychnos* by having prickles on its stem. The isolation of *S. aculeata* in its own section, *Aculeatae*, was not refuted here. Leeuwenberg (1969) suggested a possible link between *S. aculeata* and *S. camptoneura* based on morphological evidence like the arrangement of tendrils with "1-3 pairs above each other on short lateral branches." Bisset and Phillipson (1971b) strengthened the claim of a link between these two sections by noting that both species produce saponins. They also cautioned that other species may produce saponins

as these compounds have not been thoroughly investigated in this genus. The pattern of tendril placement in sections *Aculeatae* and *Scyphostrychnos* is the same in *Dolichanthae* (Leeuwenberg, 1969). Additional morphological synapomorphies for *Aculeatae* + *Dolichanthae* + *Scyphostrychnos* have not been identified yet. *Phaeotrichae*, the third monotypic section, was placed in clade IX (Fig. 3.1) where it was sister to a small American subclade.

Section *Breviflorae* is one of the largest sections in *Strychnos*, second only to sect. *Strychnos*, and is polyphyletic in this analysis. Its description incorporates a great amount of heterogenerity, for instance the tendrils may be absent, solitary or paired, the inflorescence is terminal or axillary, "stamens more or less distinctly exserted," anthers are "bearded or glabrous," and there are various types of seed testa patterns (Leeuwenberg et al., 1980). Only one specific trait in *Breviflorae's* description is invariable in the current circumscription: a short corolla tube that is glabrous inside. From our results, the length of the corolla tube and its being internally glabrous shows homoplasy when mapped onto the tree. However, Krukoff and Barneby's (1969) treatment of the American species of sect. *Breviflorae* revealed a character that is a useful synapomorphy for a portion of the species in *Breviflorae*. The American species of sect. *Breviflorae* were split into two subsections by Krukoff, subsect. *Breviflorae* and *Eriospermae*, based on characteristics of the testa. This classification was not expanded by other authors, so the African species of *Breviflorae* are not yet affiliated with either subsection. In subsect. *Eriospermae* the testa is fibrous. In subsect. *Breviflorae* the testa is crustaceous with two exceptions, *S. brasiliensis* and *S. grayii* which have a cartilagenous testa (Krukoff and Barneby 1969). The ITS data support the monophyly of

subsect. *Eriospermae* (Fig. 3.1, clade I), but subsect. *Breviflorae* is polyphyletic (Fig. 3.1, clade I and VII).

Strychnos brasiliensis was placed sister to subsect. *Eriospermae* in our ITS phylogeny, while all the other members of subsect. *Breviflorae* were included in a large poorly resolved American clade in the ITS tree (clade VII, Fig. 3.1). It would be useful to include *S. grayii*, which is restricted to Cuba and Hispaniola, in this analysis. Pending the placement of *S. grayii* it may be advisable to exclude all the species in subsect. *Breviflorae* that do not have a cartilagenous testa. This would leave only *S. brasiliensis* and *S. grayi* in subsect. *Breviflorae* and a new subsection should be created to accomodate the *Breviflorae* species with a crustaceous testa. This move could better reflect the evolution of the genus. Additional individuals of *S. brasiliensis* should be sampled as there are at least 15 synonyms associated with this name and Krukoff (1979) suspected that this species may be a widely distributed species complex.

Section *Lanigerae* is distributed in both Asia and Africa (Leeuwenberg et al., 1980). All the sampled members of this group were united into a subclade of clade IX. Its monophyly is compromised by the inclusion of *S. umbellata* and *S. axillaris* (from sect. *Brevitubae* and *Penicillatae*, respectively; (Leeuwenberg et al., 1980). Caution should be used in drawing conclusions about the Asian species and sectional affinities from the ITS data due to the more limited sampling of Asian species in this study. Of the four Asian sections, only *Strychnos* has been well-sampled by us, whereas *Brevitubae*, *Lanigerae*, and *Penicillatae* have been poorly sampled.

The remaining sections (*Brevitubae, Penicillatae, Rouhamon*, and *Strychnos*) are all polyphyletic. Floral characters were the main criteria for delineating most of the

American sections (Krukoff et al., 1942). Leeuwenberg (1969) expanded on the number and types of characters investigated, but seemingly gave some of them a lower priority when it came to developing the sectional classification. Focusing more on the wood anatomy, fruit and seed characters, and chemical data may help to identify synapomorphies for the clades recovered in this analysis. A new sectional classification including many of these new types of characters is being prepared (Frasier, in prep).

A series of preliminary recommendations for improving the sectional classification can be proposed based on ITS and morphological data. The resurrection of Progel's (1868) section *Longiflorae* is advisable, which includes the American species of section *Strychnos* sensu Leeuwenberg and Leenhouts (1980). Section *Strychnos* sensu stricto will only include the Asian species (clade VIII) and is equivalent to Hill's *Tubiflorae* (1917). A new section including *Strychnos chlorantha*, *S. colombiensis*, *S. jobertiana*, *S. panurensis*, and *S. ramentifera*, which were in section *Strychnos* sensu lato, should be considered (clade IX). The close relationship between some of these species was noted previously (Krukoff et al., 1942). The results of these changes would be the addition of two monophyletic sections, *Tubiflorae* and the *Strychnos jobertiana* group, with more morphological uniformity than is in the existing classification.

Species monophyly. To test the monophyly of species in *Strychnos*, between two and three individuals of the same species were sequenced when material was available. Most of the tested species were monophyletic, and species that were paraphyletic will not be discussed here as additional information is needed to assess their monophyly. *Strychnos angolensis* is one of the species in this analysis that was not monophyletic. This species has been described as a tree, shrub, or liana (Leeuwenberg, 1972); a

description as variable as this leaves room for misdiagnosis. Duvigneaud (1947) divided *S. angolensis* into six varieties based on leaf shape and size, as well as the length of the inflorescence and the size of the flowers; but all were climbing shrubs. *Strychnos barteri, S. darienensis, S. panamensis,* and *S. tricalysioides* were also polyphyletic, however, some of the relationships within these regions of clade II and clade VII have low support (Fig. 3.1).

Caution should be exercised when drawing conclusions based solely on ITS data. Although there are examples where concerted evolution has come to completion (Wendel et al., 1995), Álvarez and Wendel (2003) warned of complications that arise when this has not happened. The persistence of pseudogenes and the presence of more than one rDNA array all contribute to the possibility of inferring incorrect species relationships, a possibility that is increased by polyploidization (Álvarez et al., 2003). It is recommended that these data be compared to a phylogeny derived from a low-copy number nuclear gene and/or chloroplast data, as well as other independent sources like morphology.

Cytology. There is relatively little cytological data for *Strychnos*, but some patterns were observed. Cytological studies have been published for 32 *Strychnos* species, 29 of which were included in this analysis. The basic chromosome number for *Strychnos* is x=11 (Gadella, 1980). Most of the sampled species are 2n=44, with a couple of notable exceptions. *Strychnos brasiliensis* has the highest known chromosome number of the genus at 2n=110, and it is placed in clade I (Gadella, 1980). However, hybrids with a higher number of polymorphisms tend to be placed basally in consensus trees that have poor resolution (Nieto Feliner et al., 2001). *Strychnos malacoclados* and *S. angolensis*, which are morphologically similar, are the only species known to have 2n=88

and were united in the same clade in the ITS tree (Fig. 3.1, clade VII). It is not known if the polyploids are auto- or allopolyploids. A cytological study on a genus with such varying ploidy levels could be useful for tracking hybridization histories and for searching for corollaries between adaptations and ploidy number. For a more thorough discussion of ploidy levels and possible hybrids see chapter 4.

Biogeography. An in-depth biogeographic dispersal-vicariance analysis of Loganiaceae was inhibited by the lack of a well-supported and fully resolved phylogenetic tree, but some phylogeographic trends can be noted. Loganiaceae's distribution pattern is reminiscent of the Gondawanic breakup which began about 180 million years ago (mya), with South America and Africa completely separated by 90 mya (Scotese, 2004). However, Gentianales has been estimated to be from 60 my old (Muller, 1984) up to 89 my old (Wikström et al., 2001), with the younger estimate refuting the Gondwanic origin and the older estimate testing its limits. Loganiaceae was placed sister to a clade containing Gelsemiaceae and Apocynaceae by Backlund et al. (2000), and Apocynaceae is estimated to be between 45 to 53 my old (Wikström et al., 2001). Loganiaceae's age is likely similar to Apocynaceae's, and this estimate allows one to call upon the boreotropical hypotheis (Lavin et al., 1993), which describes a link between Africa and the Americas via Eurasia up until the late Eocene or the early Oligocene. A putative *Strychnos* fossil from the early Tertiary was found in west-central Oregon, USA (Chaney et al., 1933) that provides support for the boreotropical hypothesis, but upon investigation of the photograph of the fossil we have determined this not to be a *Strychnos* species (Ch. 1).

The first split in the *Strychnos* phylogenetic tree, between a tropical American clade (clade I) and an African clade (the rest), suggests a possible vicariance event separating the Latin American and African populations (Fig. 3.1). It is not possible at this point to hypothesize which region of the Americas is a potential ancestral area within clade I, since the sampled individuals were collected from Mexico to Bolivia. In the larger clade that is sister to clade I, Africa maps as the ancestral continental distribution, with subsequent dispersals to Latin America, Asia and Australia. It is not unlikely that the ancestral distribution for the genus as a whole included both Latin America and Africa, with two later dispersals from Africa to the Neotropics (clades VII and IX). Once in the Americas, there may have been a rapid radiation as the branch lengths are short and there are numerous polytomies in this region of the tree (clade VII, Fig. 3.1).

There were also two dispersals from Africa to tropical East Asia, one of which extended to Australia with *S. lucida* (clade VIII, IX). *Strychnos potatorum* was placed in clade VIII. This is the only species distributed on two continents, Africa and Asia. It was hypothesized that this species was endemic to Africa, and its distribution to Asia is the result of an anthropogenic factor (Leeuwenberg, 1969), which is supported by this study. The use of this species to clear turbid water has been recorded in Sanskrit writings (Gupta et al., 1992) and was described in the 19th century (Cooke, 1871). The seeds of this species are still in use. Seafaring travel between Africa and Asia has been occurring for hundreds, maybe even thousands, of years, which would provide opportunities for human-mediated dispersal.

Conclusions. The sections within *Strychnos* need to be reassessed so that they reflect the genus' evolution. and key characteristics should be sought by tracing

morphology and anatomy. The addition of chloroplast data would be helpful to provide a better estimate of *Strychnos* phylogeny, especially as ploidy levels may affect results based on high copy number nuclear genes like ITS.

This study is the first attempt at a *Strychnos* phylogeny and includes samples from the Americas, Africa, Madagascar, East Asia, and Australia. This includes species growing in tropical savannas and lowland rainforests, individuals from islands and mainlands. Studies on groups such as *Strychnos* that have a variable habit, broad geographical range, and high alkaloid production can provide valuable foundational knowledge for future works focusing on biogeography, morphological evolution, polyploidy, and biochemical analyses.

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Figure 3.1. Bayesian tree from ITS analysis. Posterior probabilities (pp) are above the branches; branches in bold have 100% pp. Symbols after species names indicate provenance of DNA samples: * = Americas, †= Aisa, ‡= Australia, ¤= Madagascar, and no symbol = Africa.

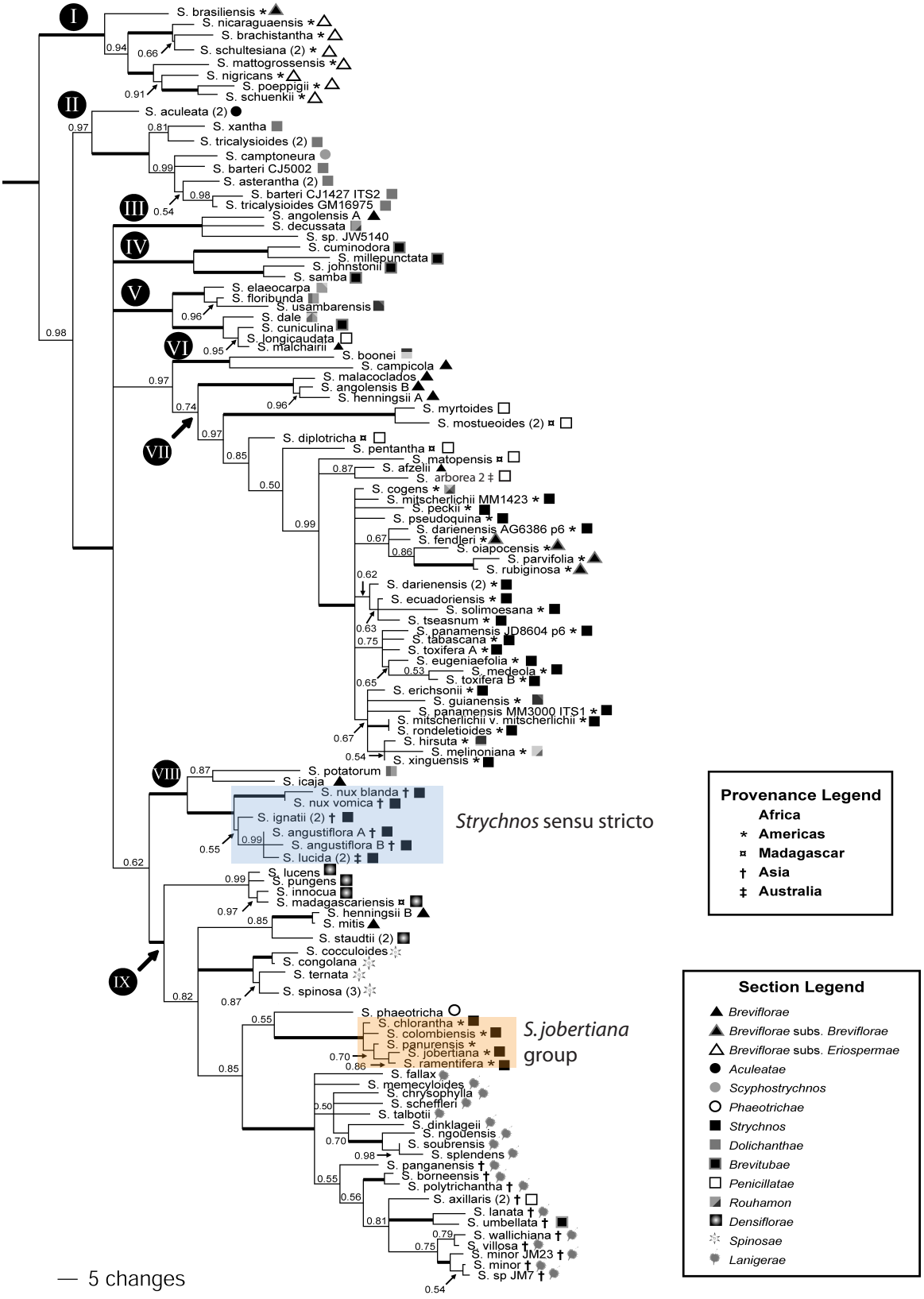
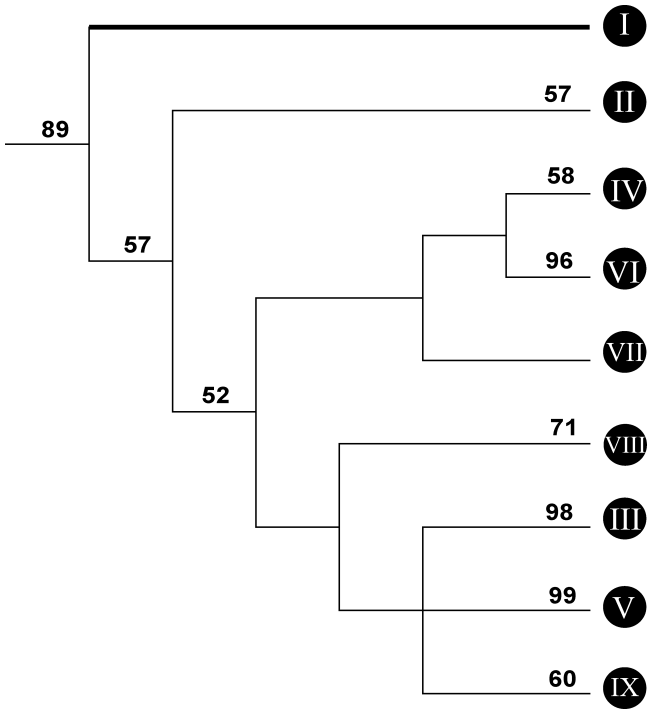


Figure 3.2. A simplified strict consensus tree from the maimum parsimony analysis of the ITS region. Jackknife (jf) support values are above the branches; branches in bold have 100% jf support.



CHAPTER 4

The History of Hybridization in *Strychnos* (Loganiaceae) as Estimated from the Internal Transcribed Spacer Region

Abstract

Strychnos has approximately 200 species distributed throughout the tropics worldwide. Recently, a phylogenetic analysis of this group based on internal transcribed spacer (ITS) sequences was presented that included more than 100 species. The variability in the placement of *Strychnos* using different gene regions from chloroplast and nuclear compartments in previous works suggest that *Strychnos* may have a hybrid origin. A close look at the ITS data revealed the possibility that additional reticulation events are currently occurring. Three sequences of putative pseudogenes were discovered and their removal from the dataset resulted in far fewer equally parsimonious trees in the MP analysis. Mapping of polymorphisms onto the phylogenetic tree revealed a subclade characterized by frequent hybridization events, with a concentration of shared polymorphisms present in three Asian species, *Strychnos umbellata*, *S. axillaris,* and *S. minor*. The latter two species have broad distributions and have been difficult to characterize due to their high morphological variability, evident by the more than 20 synonyms associated with each. The *rpl32* gene is the most suitable chloroplast region of the eight that were tested in an effort to procure an independent dataset that is not subject to the same risks associated with the ITS region.

Introduction

Polyploidy is the most common route of abrupt speciation in plants (Briggs et al., 1997), making it a critical mechanism of evolution. An estimated 50-70% of all angiosperms are estimated to be polyploids (Levin, 2000). This high percentage may be linked to polyploids' potential to insulate themselves from deleterious alleles (Otto et al., 2000) and evolve faster, because it is easier to create new genes from existing ones than *de novo* (Ohno, 1970; Wolfe, 2001). Frequently, polyploidization is a product of hybridization, and some hybrids have an increased ability to colonize a greater area than their parents (Briggs et al., 1997).

As Fuertes Aguilar and Nieto Feliner (2003) adroitly commented, "Divergence and reticulation have opposite effects in shaping the topology of phylogenetic trees." Including species that are hybrids in phylogenetic studies may potentially confound viewing the evolutionary history of a group in a tree-like format. This is of great concern as the results of almost every phylogenetic study prepared using sequence data are presented as trees. However, Soltis et al. (2008) found that the inclusion of a hybrid or an allopolyploid in a phylogenetic analysis did not significantly affect the topology of the tree, but did increase the number of shortest trees, which subsequently reduced the resolution in a strict consensus tree. The Soltis et al. study looked at the inclusion of relatively few hybrids in comparison to the total number of samples. The effects of including a greater number of hybrids may have a more significant impact on the analysis.

Currently, there are few tools available that use sequence data to investigate the origin of hybrids, but Baldwin (1995) commented on the possibility of the internal

transcribed spacer (ITS) region of the nuclear ribosomal DNA being useful for exploring the parentage of hybrids. Since then, ITS data has provided information on the polyploid origins of *Lycoris*: Amaryllidaceae (Shi et al., 2006), *Malus*: Rosaceae (Feng et al., 2007), *Armeria*: Plumbaginaceae (Fuertes Aguilar et al., 2003), and others.

Since ITS is in the nuclear genome, it is biparentally inherited. It is a high-copy number region that occurs in tandem repeats, with thousands of copies within one individual's genome on multiple chromosomes (Baldwin et al., 1995). Concerted evolution is a mechanism that homogenizes the ITS copies via various crossing over incidences during meiosis, and has been proposed to be the driving force behind the uniformity of the ITS region. However, concerted evolution is an ongoing process and numerous examples of it not coming to completion have been discovered (Álvarez et al., 2003). Hybridization can have three chief consequences on ITS sequences: additive patterns where both parental sequences are represented (Soltis et al., 1991), the formation of a chimeric sequence (Nieto Feliner et al., 2004), or biased homogenization leading to the loss of one of the parental ribotypes (Fuertes Aguilar et al., 2003). The loss of one of the parental lineages will not be useful when trying to reconstruct the origin of a hybrid, but the maintenance of both ribotypes is sometimes evident as polymorphic sites in the ITS sequence data (Fuertes Aguilar et al., 2003; Soltis et al., 2008). These additive polymorphic sites (APS) can be scored and used to trace the potential parents. This process may be useful for tracking the origin of putative *Strychnos* (Loganiaceae) hybrids as identified in this study.

The varying ploidy numbers in *Strychnos* open the door to the possibility of the existence of hybrids. *Strychnos* is the most species-rich member of Loganiaceae

(Gentianales) with approximately 200 species globally distributed throughout the tropics. Cytotaxonomic data from this genus suggests that the basic chromosome number is x=11 (Gadella, 1980). Of the 32 species whose chromosomes have been counted, the majority are 2n=4x=44, suggesting that many *Strychnos* species are tetraploids. Two species, *Strychnos angolensis* and *S. malacaclados*, have 2n=8x=88. *Strychnos brasiliensis*, a neotropical species, has the highest recorded chromosome count in the genus at 2n=10x=110, making it a decaploid (Gadella, 1980).

Once the basic chromosome number for a group has been determined, the pattern in a polyploid series can be seen. For example, *Strychnos* would have 2n=2x=22, 2n=3x=33, 2n=4x=44, and so on. However, the first steps in this polyploid series have not been observed in *Strychnos*. This may be a sign that *Strychnos* is an ancient polyploid and the lower portions of the series have since gone extinct (Briggs et al., 1997) or it might be due to poor sampling. Also, odd numbers in the series frequently result in sterile hybrids, possibly explaining the lack of these steps in the series.

Although numerous *Strychnos* species have been known to be polyploids since the mid 20th century (Mangenot et al., 1957; Raghavan, 1959; Miège, 1960; Gadella, 1962), there is only one published account describing three potential hybrids between closely related African species (Leeuwenberg, 1969). *Strychnos* species are valuable as the source of numerous traditional medicines and these ethnobotanical uses have drawn many researchers to the genus, which has resulted in the publication of several chemical (Marini-Bettòlo et al., 1967), morphological (Krukoff et al., 1942; Leeuwenberg, 1969; Bisset et al., 1973), and phylogenetic (Ch. 3) studies. Additionally, distribution maps exist for many African species of *Strychnos* (Leeuwenberg, 1969), which will be useful

when estimating the plausibility of potential parentage. Any study that propels the understanding of this genus' evolutionary history is valuable for accomplishing one more step in preparing the foundation for estimating the effects of polyploidy on chemistry, ecology, and morphology.

A phylogenetic investigation of *Strychnos* using the nuclear ribosomal internal transcribed spacer (ITS) region was recently conducted (Ch. 3). Twenty-nine of the 32 species with known chromosome counts were included in this analysis, along with more than 70 other species. Molecular data from multiple unlinked loci is preferred to reconstruct the phylogenetic relationships between taxa to prevent basing all conclusions on a gene tree that does not reflect the species tree (Nichols, 2001). In light of this, sequences from a chloroplast gene would be valuable to corroborating relationships and identifying hybrids which can have conflicting placement in trees between different genes. The objectives of this study are to predict which *Strychnos* species may be allopolyploids using the ITS sequence data, determine the parentage of these hybrids, and suggest a chloroplast gene for further studies. Herbarium specimens will be referenced to see if there are visible effects of polyploidy such as the presence of the 'gigas' phenotype (Lewis, 1980), a higher degree of morphological variability, and a broader distribution (Briggs et al., 1997).

Materials and Methods

The ITS sequences were products of a previous study and were sequenced in both directions with primers that nearly entirely overlapped (Ch. 2 and 3). Additive polymorphisms were identified by comparing all the chromatograms involved in

obtaining a complete ITS sequence. The criteria of Fuertes Aguilar et al. (1999) and Fuertes Aguilar and Nieto Feliner (2003) were followed and are described here briefly. Sites were deemed to be polymorphic only if the competing signals were detected in the forward and reverse primer sequences and the weakest signal was at least 25% the strength of the stronger signal. All polymorphisms were recorded, but only APS are useful for estimating parentage. Additive polymorphisms are sites where the bases involved are also represented individually in the same position in other accessions of the ITS dataset. For a more complete description see Fuertes Aguilar and Nieto Feliner's publication (2003).

Pseudogene detection. When working with ITS sequences, especially from putative hybrids, cloning of the PCR products should be done. This is to check for sequence variability within an individual, which is important for detecting pseudogenes. Pseudogenes can violate the principle of homology followed in phylogenetic analyses. Since cloning was not performed, alternative methods of pseudogene detection were pursued. The GC content of pseudogenes is typically lower than non-pseudogenes, so all sequences were inspected for aberrant GC levels. Additionally, pair-wise base differences were calculated using PAUP, and sequences that were one standard deviation from the norm were marked as possible pseudogenes. Lastly, the secondary structure of the ITS region may not be conserved in pseudogenes, which could result in higher minimum free energy (mfe) values due to a lack of selection pressure. This last criterion was given the highest priority, because the inability for the ITS region to fold into a specific secondary structure suggests that the gene is not functional. The mfe values were calculated in an RNAsalsa analysis performed in Frasier and Struwe (Ch. 3).

Additional signs of secondary structure disruption were sought in suspect sequences by examining the postscript files also produced by RNAsalsa. These show a graphical depiction of the structure of the ITS region that was predicted for each individual taxa. If a sequence had any combination of these three characteristics, it was excluded from further analysis.

Phylogenetic analysis. Bayesian and maximum parsimony (MP) phylogenetic analyses of the ITS dataset were performed, but unlike in Frasier and Struwe's (Accepted) previous work the pseudogene sequences were excluded. MrBayes 3.1.2 (Huelsenbeck et al., 2001) was used to perform the Bayesian analysis with the GTR+I+G model, as this was selected by the Akaike Information Criterion via MrModeltest v2 (Nylander, 2004). PAUP (Swofford, 2002) was used to conduct the MP analysis, and jackknife values were obtained via 200 replicates each with 1000 random addition replicates.

Splitstree4 (Huson et al., 2006) was used to calculate a splits graph using the neighbor-net algorithm (Bryant et al., 2004) from the uncorrected pairwise distances. Incomplete sequences were excluded as distance methods have been suspected to be more sensitive to missing data (Gatesy et al., 2002), although Wiens (2003) found this not to be the case in a limited study.

rpl32. A small scale analysis using *rpl32* sequences from the chloroplast genome was also performed to determine if the clades resolved by the ITS data would be supported by an independent data source. At least two representatives from each clade were sequenced when possible, for a total of 22 *Strychnos* taxa. PCR reactions for the *rpl32* region were prepared the same as for the ITS region (Ch. 2). The forward primer

and thermocylcing program of Shaw et al. (2007) were used plus a reverse primer designed for *Strychnos*: CCCATCCACCTATTTATTACAA. Bayesian and MP analyses were conducted on the first half of the gene region to compare the results to a previous study (Ch. 3).

Results

Pseudogene detection. The mean base frequencies for 129 *Strychnos* samples were A=0.197, C=0.324, G=0.304, and T=0.176. It is common for the ITS region to have a higher GC content than AT. A partial ITS sequence was obtained for *Strychnos trichocalyx*, and this had the lowest cytosine content of all samples, C=26.3%, but its guanine content was close to average, G=29.4%. It also had the greatest pairwise base difference. The secondary structure was well-supported for ITS2; ITS1 was not successfully sequenced. Since *Strychnos trichocalyx* had two out of three pseudogene characteristics, it was removed from the phylogenetic analysis.

The ITS regions of two individuals of *Strychnos mosteuoides* deviated from the other *Strychnos* species in a well-conserved region that stretches from the very end of 5.8S through stem 2A of ITS2 (Ch 2). Non-synonymous mutations in conserved regions is a symptom of a pseudogene (Grimm et al., 2008) as well as the disruption of the secondary structure, so these taxa were also excluded from the analysis.

Additive polymorphic sites. Chromatograms for all *Strychnos* samples were searched for the presence of APS. Polymorphisms were detected in 87 out of 130 samples, suggesting that the majority of the sampled *Strychnos* species are hybrids. There are species that have polymorphisms, but were not additive according to the

current sampling. Of the 87 accessions that have polymorphisms, 41 have additive polymorphisms. The positions of all polymorphisms were recorded, for a sample see table 4.1.

Both individuals of *Strychnos angustiflora* share a polymorphism, and three Filipino *Strychnos minor* individuals share many APS. Closely related species that share APS are *Strychnos xantha* and *S. tricalysioides* (clade II), *S. cuniculina* and *S. malchairii* (clade V), and *S. chlorantha* and *S. jobertiana* (clade IX). Other species also share APS, but are in different clades. For example, Strychnos *phaeotricha* (clade IX) shares the same APS as *S. tricalysioides* and *S. xantha* (clade II).

Phylogenetic analysis. After the exclusion of the possible pseudogenes, there were far fewer most parsimonious trees, 4243 (L=1579 steps, CI=0.40, RI=0.74) versus 6289 trees (L=1623 steps, CI=0.40, RI=0.73). The topology only varied in the region of clade VII in both the Bayesian and MP trees, which had the lowest branch support of the nine delineated clades. The removal of *Strychnos mosteuoides* resulted in the fragmentation of clade VII into three units with *Strychnos myrtoides* sister to clade III, and the other two fragments in a polytomy with clade VI (Fig. 4.1). See figure 4.2 for a close-up of a subclade with shared polymorphisms marked on the branches.

There was little variation in the placement of putative hybrids in the 20 MP trees that were randomly selected for observation. Nearly all the differences between trees were concentrated in the large American group of species in clade VII, where the strict consensus tree lacks resolution.

Ninety-five of the 126 *Strychnos* sequences were included in the splits graph (Fig. 4.3), approximately 75% of the data. The clades in the ITS tree were readily identified,

but certain portions of the network are characterized by many competing topologies, especially clades VII and IX. The phylogenetic signals in these two clades were incompatible and resulted in decreased resolution in the ITS tree, which is clearly evident in the American subclade (clade VII). The red edges in the network are those that lead to an Asian subclade with numerous putative hybrids (Fig. 4.3). Portions of the network were treelike, such as clades I, II, IV, V, and to a lesser extent, VIII.

rpl32. The *rpl32* region was simple to align and done so using the alignment utilities built in to the Sequencher program. *Antonia ovata* was included in a preliminary analysis of the *rpl32* data to help root the tree. This was not included in the final analysis because the dataset was truncated due to ambiguous alignment of a portion of *Antonia*'s sequence with the ingroup. In this preliminary analysis, *Strychnos tricalysioides* and *S. aculeata* were placed basal to the remainder of the *Strychnos* species. The GTR+G model was used for the Bayesian analysis of the *rpl32* data. The MP analysis yielded 158 trees (L=193, CI=0.83, RI=0.62).

Clades I, II, and V from the ITS tree were also supported in the *rpl32* tree (Fig. 4.4). The close relationship of clades I and II was also recovered. Placement of *Strychnos brasiliensis* in clade I was reinforced. Weakly supported sister relationship between *Strychnos boonei* with *S. ecuadoriensis* (clade VI and VII in the ITS tree) and a clade containing *S. diplotricha*, *S. phaeotricha*, and *S. splendens* (clades VII and IX in the ITS tree) were not in agreement with the ITS data (Fig. 4.1 and 4.4). The remainder of the taxa were in a polytomy, and neither supported or refuted any relationships suggested by the ITS data. The *rpl32* region shows promise as a possible chloroplast candidate

gene for developing an additional phylogeny that should not have the same hazards as the ITS region.

Discussion

Analysis of at least five different unlinked gene regions is recommended to detect a given reticulation event with 95% confidence (Rieseberg et al., 1995). This has been done for Loganiaceae with five chloroplast genes, *matK*, *ndhF*, *rbcL*, *rps16*, and *trnL*, and one nuclear gene, ITS (Ch.1 and 2). The variability in the placement of *Strychnos*, *Gardneria*, *Neuburgia*, and *Spigelia* suggest that an ancient hybridization event is complicating the phylogenetic assessment of the family.

There is no cytological data currently available for *Gardneria* or *Neuburgia*, but both *Strychnos* and *Spigelia* include polyploid species. *Strychnos* has a basic chromosome number of x=11 and tetraploids, octaploids, and a decaploid have been reported (Gadella, 1980). *Spigelia* has a basic chromosome number of x=8 and has tetraploids, hexaploids, and octaploids (Gadella, 1980). No diploids have been recorded for either genus, but only a few species have been cytologically examined.

A possible scenario is that two independent hybridization events gave rise to *Strychnos* and *Spigelia*. A plausible parent for *Strychnos* is *Usteria* as this genus grows as a liana or scandent shrub like most *Strychnos* species and is distributed in Africa (Leeuwenberg et al., 1980), the possible cradle for *Strychnos* (Ch. 3). Additionally, *Usteria* is a diploid with a basic chromosome number of x=11, but this is the only genus of Antonieae for which a chromosome count is available. The other parental lineage is more obscure, or perhaps a chromosome doubling event may have occurred creating an

immediate reproductive barrier (Ramsey et al., 1998), and subsequent mutations gave rise to *Strychnos*. *Spigelia* is morphologically similar to *Mitreola*, so much so that *Mitreola* has been included in Spigelieae (Solereder, 1892-1895; Leenhouts, 1963; Leeuwenberg et al., 1980) and *Spigelia* has been included in Loganieae with *Mitreola* (Bentham, 1856; Bentham et al., 1876). *Mitreola* is a diploid with a basic chromosome number of x=10. *Spigelia* is always placed near to the members of Strychneae (Ch. 1 and 2), suggesting that they should be investigated as candidates for parentage although there is little morphological similarity.

The majority of *Strychnos* species had polymorphisms in their ITS sequences, approximately half were APS. However, it was not possible to estimate the parentage of these species as frequently there were too many individuals that could be the donor of the bases in question. A preliminary review of morphological characters did not significantly narrow the possible parental pool, but due to the large number of hybrid taxa, a thorough morphological analysis would require a great deal of time. Although parentage could not be determined, there were three prominent examples of the inheritance of polymorphisms in *Strychnos* species that demonstrated that some taxa share a hybrid ancestor. The first was a polymorphism that is shared between the sister taxa *Strychnos tricalysioides* and *S. xantha* of clade II at base 139. Both these species show little evidence of hybridization as they have limited geographical distributions and restricted morphological variability.

Strychnos minor and *S. axillaris* are two species that do have the aforementioned hybrid characteristics. *Strychnos minor* is a broadly-distributed species found in India, Myanmar, Thailand, Vietnam, Malaysia, Borneo, the Philippines, Solomon Islands, and onto Australia (Bisset et al., 1973). This species is associated with at least 26 synonyms

(Bisset et al., 1973; Conn et al., 1993). The taxa that were reduced to synonyms of *Strychnos minor* were from different sections in the genus (Hill, 1917). Since the sections were circumscribed using morphological characters, the combination of these diverse entities into one species resulted in a morphologically variable unit. This variability is readily observed in herbarium collections. Like *Strychnos minor*, *S. axillaris* also has a broad distribution throughout continental southeast Asia and Malesia, but does not extend into Australia (Bisset et al., 1973; Conn et al., 1993). *Strychnos axillaris* has been difficult to characterize due to its morphological variability and has approximately 27 synonyms (Tirel-Roudet, 1970; Bisset et al., 1973).

Strychnos minor and *S. axillaris* are in a highly derived subclade of clade IX. All Filipino *Strychnos minor* individuals have the same polymorphic base call at position 444 and two have the same polymorphism at base 388 (Fig. 4.2). The sequences of the individuals of this species seem to be becoming uniform at different rates; the three Filipino *Strychnos* species have between two to six polymorphic base calls each. A *Strychnos minor* individual from the Solomon Islands did not share these feature and was placed sister to *S. vitiensis* from Fiji, making the species paraphyletic. The difficulties encountered with circumscribing this species are likely linked to a relatively recent hybrid origin.

Two individuals of *Strychnos axillaris*, both in clade IX, were sampled. Like one of the *Strychnos minor* individuals, they also have a polymorphic base call at position 454. For *Strychnos axillaris*, both adenine and thymine were present, but the *S. minor* individuals had cytosine and thymine. An ancestor with thymine in this position may be shared between the two clades, with separate subsequent hybridization events occurring

or substitution at this site. Similarly, the broad range, variable features, and polymorphic base calls support the hybrid origin of *Strychnos axillaris*.

Strychnos umbellata, also an Asian species in the same subclade as *S. minor* and *S. axillaris*, had polymorphic base calls. A polymorphism at base 388 is shared by *Strychnos umbellata* and the two Filipino individuals of *Strychnos minor*, and a polymorphism at base 492 is shared between *S. umbellata*, and one of the *Strychnos minor* (ISU49) individuals (Fig. 4.2). This data supports the idea that there was a hybridization event unique to this subclade containing *Strychnos axillaris*, *S. minor* and *S. umbellata*, and that concerted evolution has not come to completion. There are four other species in this subclade that do not have polymorphisms. Concerted evolution may have come to completion in these individuals, or a bias in the PCR reaction prevented one of the ribotypes from being amplified (Suzuki et al., 1996).

A small American subclade is also present in clade IX. Two of the five individuals (*Strychnos chlorantha* and *S. jobertiana*) share a polymorphism at position 347. There are very few polymorphisms coded for this group, only one to two per individual. *Strychnos chlorantha* is a large liana from central America, and *Strychnos ramentifera*, also a large liana, has a more southern distribution in the Amazon Basin (Krukoff, 1972). They have somewhat restricted distributions, relatively few polymorphisms, and neither species show obvious gigas characteristics. The possibility of a hybrid origin for this small subclade of American species should be investigated more thoroughly.

In general, there was a higher concentration of putative hybrids in clade IX, 20 out of 37 taxa, with the majority of hybrids in the more derived branches. This may be a

sign that these taxa have less stringent reproduction barriers between species. *Strychnos axillaris* and *S. minor* may be relatively young species that are subject to active evolutionary processes. They are distributed on islands and mainlands and repeated contact between these secluded populations may result in morphological patterns that are difficult to interpret (Frasier et al., 2008).

Strychnos brasiliensis, a polyploid with the highest chromosome number in the genus (Gadella, 1980), is similar to *S. axillaris* and *S. minor* in that it has a broad geographical range, southeastern Brazil, Paraguay, Argentina, and Bolivia (Krukoff, 1972), and it is also morphologically variable. Krukoff and Monachino described the species as a "complex with a multitude of forms and races (1942)." *Strychnos brasiliensis* was in a basal position in the ITS tree (Fig. 4.1), which occasionally happens with hybrids that have many polymorphisms (Nieto Feliner et al., 2001). Therefore, the absence of polymorphisms from the ITS sequence was unexpected. The relationship of *Strychnos brasiliensis* to *S. mattogrossensis* and *S. poeppigii* was also recovered in the *rpl32* tree (Fig. 4.3). Perhaps the consistent placement of *Strychnos brasiliensis* in both gene trees and the absence of polymorphisms could be explained by chromosome doubling after the initial ancient hybridization event. This species could be a 'fixed' older lineage, and may benefit from a traditional alpha-taxonomic treatment.

The *rpl32* tree supports multiple clades from the ITS tree contributing additional evidence in favor of those relationships (Fig 4.1 and 4.4). There were two instances of disagreement, but neither was well-supported. The *rpl32* data are preliminary, and a much broader sampling is necessary before drawing conclusions. The use of this gene is recommended for additional molecular phylogenetic studies as it outperformed seven

other candidate genes (trnS-trnG, trnG intron, trnC-trnD, trnS-trnfM, trnH-psbA, rps16, and rpl16).

Unlike the Soltis et al. (2008) study, this work contains a majority of possible hybrids. In this case, using a network as well as the conventional tree-like format is more informative (Fig. 4.1 & 4.3). Some of the clades delineated according to the ITS tree are believed to reflect the true evolutionary relationships within *Strychnos*; they are supported by morphology (see Chapter 2 more information) and in certain cases, by the *rpl32* data (clades I, II, and V). Clade VII, which had lower branch support than the others, may be better interpreted as two smaller monophyletic associations as the unit splintered after the removal of the pseudogenous *Strychnos mosteuoides* sequences, and there are two clear subdivisions in the splits graph (Fig. 4.3). Although no conclusions could be drawn concerning the parentage of the hybrids, an analysis of polymorphisms shed light on the challenges associated with delineating two widespread variable species, *Strychnos axillaris* and *S. minor*, and the inheritance of polymorphisms suggested that concerted evolution has not come to completion in many taxa.

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Table 4.1. Sample of polymorphisms from 17*Strychnos* accessions out of 102. Shared polymorphisms are present in multiple accessions of*Strychnos angustiflora* , *S. minor* , and *S. staudtii* species.

		Position in alignmnent								
Species	**Clade**	**139**	**289**	**347**	**388**	**444**	**489**	**492**	**522**	**559**
Strychnos afzelii	VII		R							
Strychnos angustiflora 1	VIII								M	
Strychnos angustiflora 2	VIII								M	
Strychnos axillaris 1	IX						R			
Strychnos chlorantha	IX			S						
Strychnos jobertiana	IX			S						
Strychnos minor 1	IX				Y	Y	K	R		
Strychnos minor 2	IX					Y				
Strychnos minor 3	IX				Y	Y	K			
Strychnos phaeotricha	IX	S								
Strychnos staudtii 1	IX									Y
Strychnos staudtii 2	IX									Y
Strychnos talbotiae	IX						R			
Strychnos tricalysioides 1	II		R							
Strychnos tricalysioides 2	II	S								
Strychnos umbellata	IX				Y			R		
Strychnos xantha	II	S								

Figure 4.1. Strict consensus Bayesian ITS tree after removal of putative pseudogenes Numbers on clades are those used in Ch 3, and numbers on branches are jackknife values Clade VII is not supported as a monophyletic group after the removal of pseudogenes.

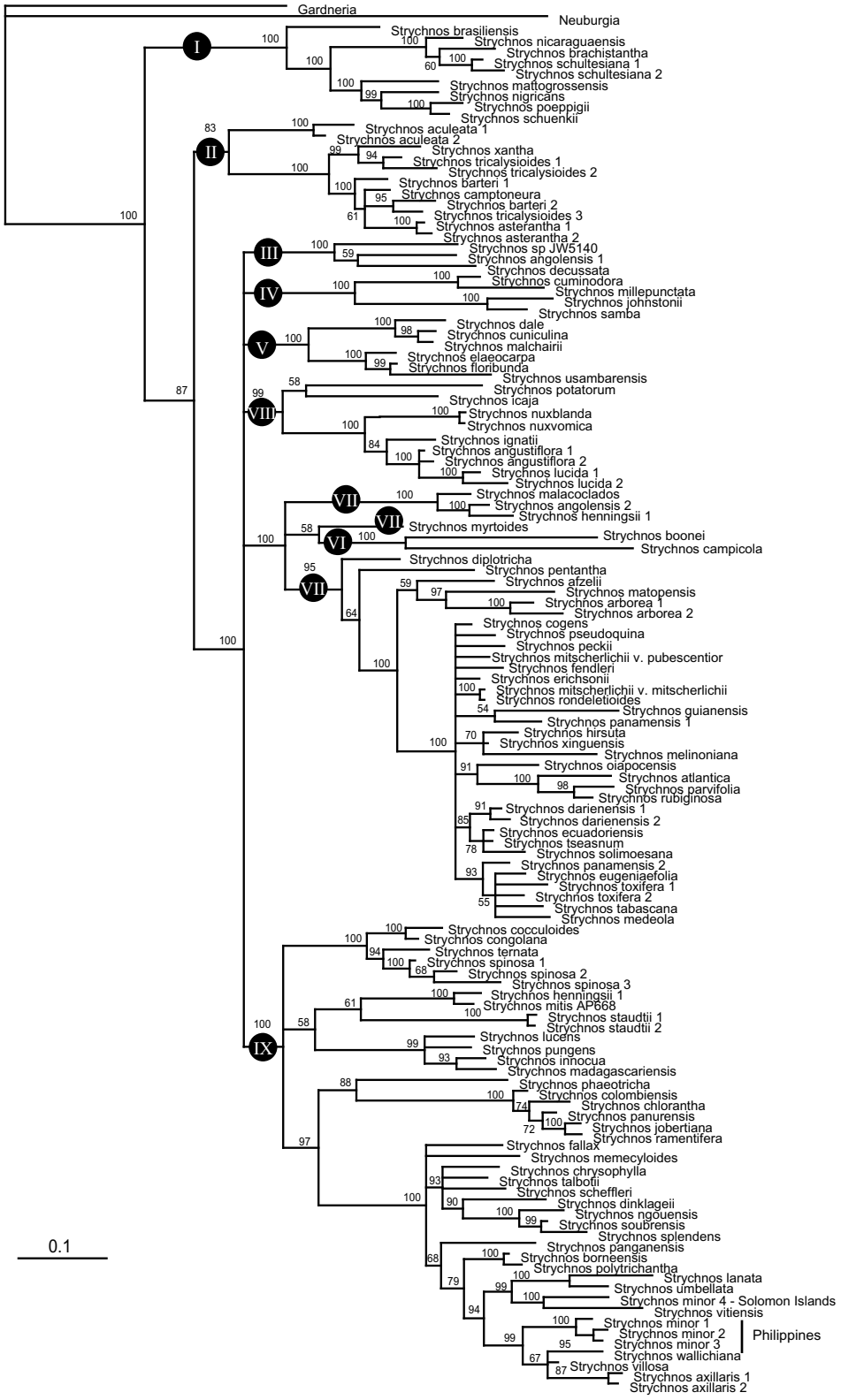


Figure 4.2. A closeup of the region of a subclade of clade IX with polymorphisms that are shared between taxa marked with colored rectangles on the branches of the tree.

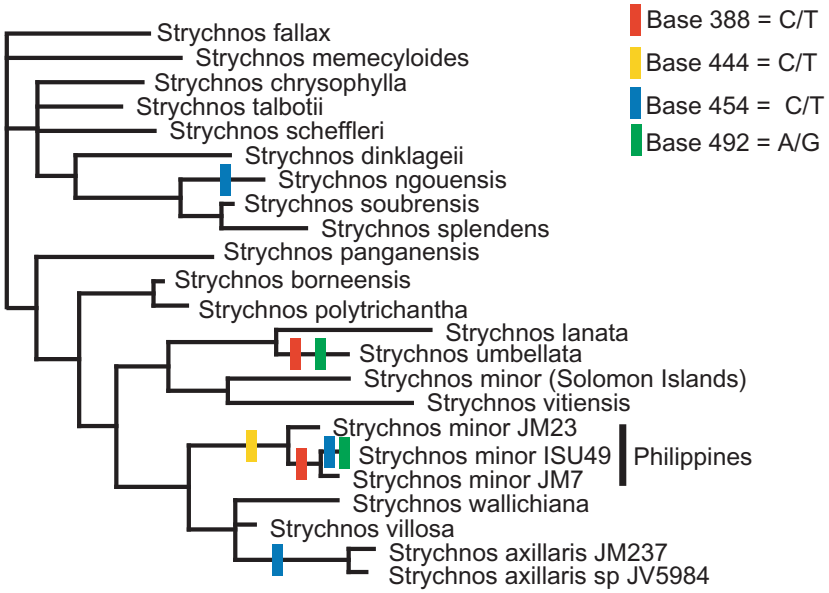


Figure 4.3. Neighbor-net splits graph based on uncorrected pairwise distances inferred from the original data. Pseudogenes and incomplete sequences have been excluded. Roman numerals correspond to clades from the ITS tree. Red edges indicated an Asian subclade containing numerous putative hybrids that share polymorphisms..

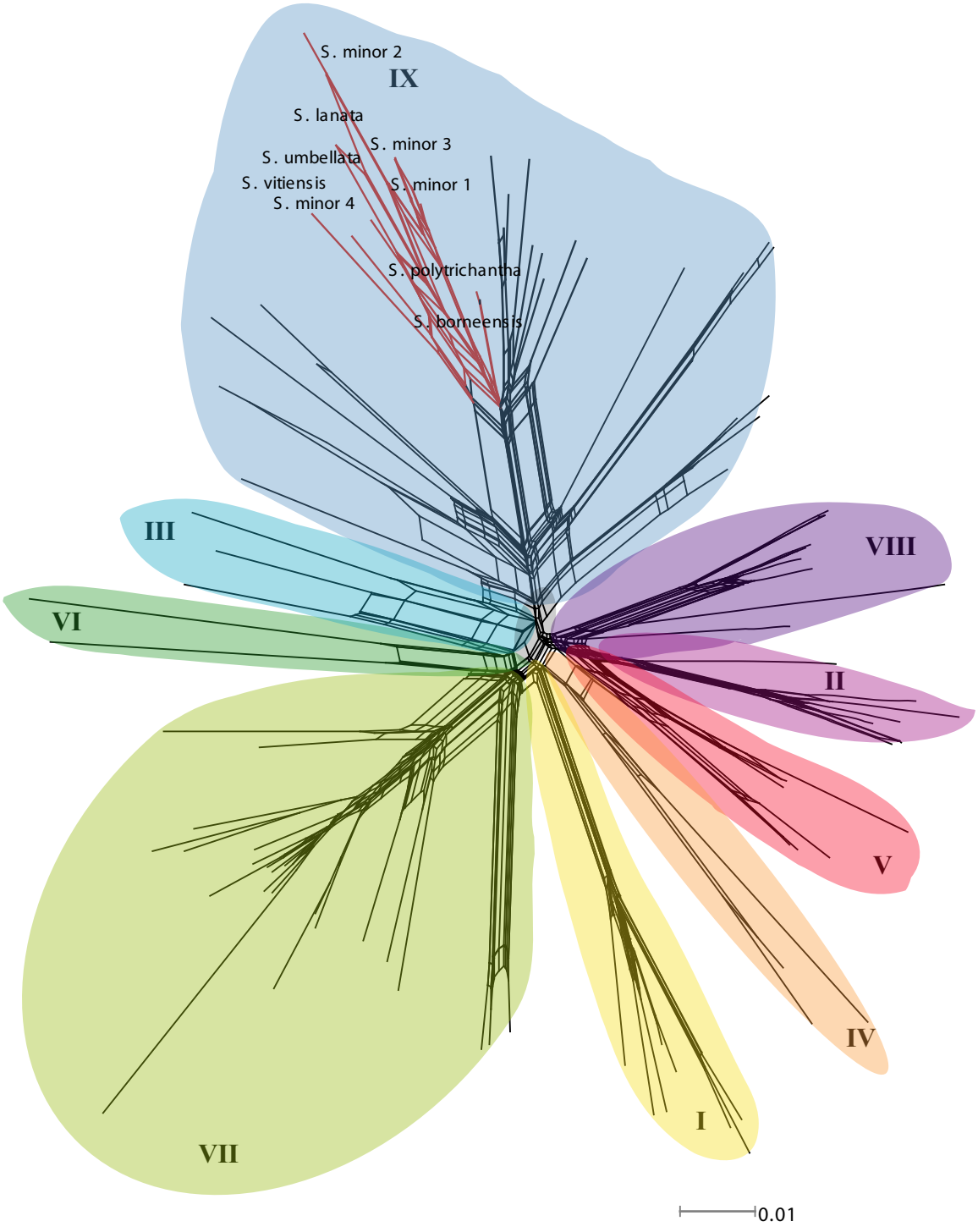
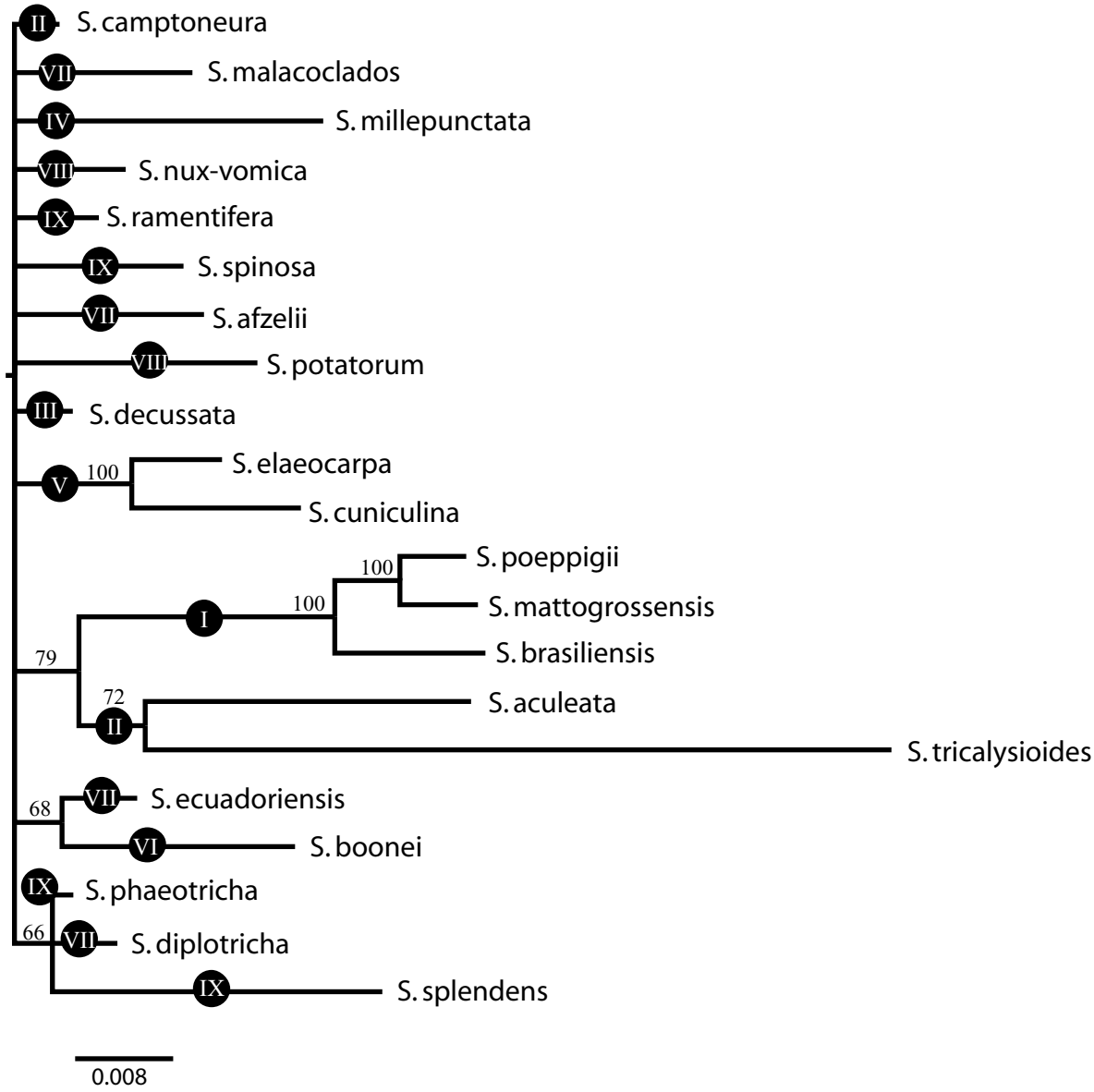


Figure 4.4. Bayesian tree of the rpl32 region with pp values on branches. Numbers in circles correspond to clades in the ITS tree.



Appendix 1. GenBank accession numbers for all sequences used in the study.

		GenBank accession number			
Family	**Taxa**	**matK**	**ndhF**	**rbcL**	**trnL**
Apocynaceae	*Alstonia scholaris*	Z70189	AJ011982	X91760	
	Alstonia boonei				AF102374
	Apocynum androsaemifolium	EF456263			
	Apocynum cannabinum			X91761	AF102380
	Araujia sericifera	Z98194	AF130165	AJ419734	DQ221129
	Kopsia fruticosa	Z70178	AJ235824	X91763	
	Nerium oleander	AY899942	AF130168	AF156735	AF214386
	Periploca graeca	Z98178	AJ235825	AJ002889	AF214244
	Secamone oleifolia	AY899954			AF214421
	Thevetia peruviana	Z70188	AF130169	X91773	AF214436
	Wrightia arborea			AJ002891	
	Wrightia dubia	EF456257			
	Wrightia tomentosa				AF214453
Gelsemiaceae	*Gelsemium sempervirens*	AJ429322	AJ011984	L14397	
	Mostuea brunonis		AJ235828	L14404	
	Mostuea hirsuta	EF077194			
	Mostuea surinamensis				BVR2318
Gentianaceae	*Anthocleista grandiflora*		AJ235829	L14389	
	Anthocleista scandens				AF102376
	Anthocleista schweinfurthii	EF077197			
	Blackstonia imperfloiata	This study	This study	This study	AF102384
	Curtia tenuifolia		This study	This study	This study
	Exacum affine		AJ011983	L11684	AF102417
	Fagraea berteroana	This study			AF102419
	Fagraea sp.			L14396	
	Gentiana frigida				AF102435
	Gentiana procera		L36400	L14398	
	Gentiana purpurea	AJ429323			
	Irlbachia pratensis		This study	This study	AF102442
	Lisianthius jefensis		This study	This study	This study
	Macrocarpaea glabra		This study	This study	This study
	Potalia resinifera	This study	AJ235831	AJ235816	AF102472
	Schultesiana guia		This study	This study	This study
	Swertia perennis	This study	This study	This study	This study
Loganiaceae	*Antonia ovata*		AJ235832	AJ235817	AF102379
	Bonyunia aquatica	This study			
	Bonyunia minor		AJ235833	AJ235818	This study
	Gardneria angustifolia		AJ235834	AJ235819	
	Gardneria multiflora	This study			
	Gardneria ovata				This study
	Geniostoma rupestre	Z70194	AJ235835	Z68828	AF102430
	Labordia kaalae	This study			
	Labordia tinifolia		AJ235836	AJ235820	AF102447
	Logania albiflora				AF102451
	Logania vaginalis	AJ429324	AJ235837	Z68826	

		GenBank accession number			
Family	**Taxa**	**matK**	**ndhF**	**rbcL**	**trnL**
	Mitrasacme pilosa		AJ236058	AJ235821	AF102459
Loganiaceae	*Mitreola petiolata*		AJ235839	AJ235822	AF102460
	Neuburgia corynocarpa		AF027275	AJ001755	AF102462
	Neuburgia neocaledonica	This study			
	Spigelia anthelmia	This study	AJ235840		This study
	Spigelia marilandica			L14007	
	Strychnos nux-vomica	Z70193		L14410	AF102485
	Strychnos potatorum		AJ235841		
	Usteria guinenensis		AJ235842	AJ235823	AF102496
Rubiaceae	*Cephalanthus occidentalis*	AY538377	AJ236288	X83629	AF152692
	Chiococca alba	AY538378		L14394	AF102400
	Chiococca race		AJ130835		
	Cinchona officinalis	AY538381			
	Cinchona pitayensis				AF152684
	Cincchona pubescens		AJ235843	X83630	
	Erithalis fruticosa		AJ236295	X83635	AF152697
	Gardenia taitensis				AF102426
	Gardenia thunbergia	Z70198	AJ235844	X83637	
	Guettarda ferruginea				AF152731
	Guettarda speciosa	AY538389			
	Guettarda uruguensis		AJ236297	X83638	
	Ixora coccinea	AM412468	AJ236299	X83646	
	Ixora killipii				AF152659
	Mussaenda erytrophylla		AJ130836	X83652	
	Mussaenda hybrid cultivar				AF152651
	Mussaenda raiateensis	AY538406			
	Ophiorrhiza mungos	AY538408	AJ130838	X83656	AF152610
	Pentas lanceolata	AB247151	AJ236304	X83659	
	Pinckneya pubescens		AJ130839	X83661	AF152648
	Rogiera suffrutescens	AY538419	AJ236308	X83665	
	Rondeletia odorata		AJ235845	Y11857	AF152741
	Vangueria edulis	AY538424			AF152654
	Vangueria mada		AJ130840	X83670	

Appendix 2. GenBank accession numbers for all outgroup sequences used in this study.

			GenBank accession number			
Order	**Family**	**Taxa**	***matK***	***ndhF***	***rbcL***	***trnL***
Cornales	Hydrangeaceae	*Hydrangea macrophylla*	AB236030	AF130218	L11187	
		Hydrangea paniculata				X85796
Dipsacales	Adoxaceae	*Viburnum acerifolium*	AF446897		L01959	
		Viburnum rhytidophyllum		AF027273		
	Valerianaceae	*Valeriana fauriei*		AF130192		
		Valeriana officinalis	AY310467		L13934	AF366917
Lamiales	Oleaceae	*Jasminum mesnyi*		AF130162		
		Jasminum nudiflorum	AF531779			
		Jasminum officinale				AF231843
		Jasminum suavissimum			L01929	
		Olea europaea	AM229542	AF027288	AJ001766	AF231866
	Plantaginaceae	*Antirrhinum majus*	AF051978	L36392	L11688	AF118790
	Verbenaceae	*Verbena bracteata*		L36418		
		Verbena officinalis			Z37473	AF231885
		Verbena rigida	AJ429353			
Solanales	Solanaceae	*Nicotiana tabacum*		Z00044	Z00044	Z00044
		Petunia axillaris	AJ585880	U08926		
		Petunia x hybrida			X04976	X74572

Appendix 3. Voucher information for sequences generated for this study and accession numbers for sequences taken from GenBank.

	Voucher Information:	**GenBank accession #**	
Taxa	Collector name, number (voucher herb)	**ITS**	**rps16**
Antonia ovata Pohl.	S. Mori 24159 (NY)	This study	AF004091
Bonyunia aquatica Ducke	P. Berry et al. 5771 (MO)	This study	
Bonyunia minor N. E. Br.	P. Berry and L. Brako 5522 (NY)	This study	This study
Gardneria multiflora Makino	K. Yao 11481 (NY)	This study	This study
Gardneria ovata Wall.	J. Klackenburg and R. Lundin 214 (NY)	This study	
Geniostoma rupestre J.R.Forst. & G.Forst.		DQ499095	This study
Labordia kaalae C. N. Forbes	T. Motley 1203 (BISH)	This study	This study
Logania albiflora (Andrews) Druce		DQ358879	
Logania vaginalis (Labill.) F. Muell.			AJ431035
Mitrasacme polymorpha R. Br.	Anonymous 20495 (NY)	This study	This study
Mitreola petiolata (Gmel.) Torr. & A. Gray		AF054635	
Neuburgia celebica (Koord.) Leenh.	T. Flynn 6303 (NY)	This study	
Neuburgia novocaledonica (Gilg & Benedict) J. Molina & Struwe	L. Struwe 1301 (NY)	This study	This study
Norrisia malaccensis Gardner	B. C. Stone 14107 (HUH)	This study	This study
Spigelia anthelmia L.		This study	
Spigelia coelostylioides K.R. Gould		AF177992	
Spigelia hedyotidea K.R. Gould		AF178008	
Spigelia loganioides A. DC.		AF178000	
Spigelia marilandica L.		AF177991	
Spigelia texana A. DC.		AF178006	
Spigelia sp.			AF004093
Strychnos brasiliensis Mart.	C. Medri et al. 446 (NY)	This study	
Strychnos nux-vomica L.	J. F. Maxwell 90-622 (MO)	This study	AF004094
Strychnos spinosa Lam.	T. Motley - from Coral Gables Bot Gard., FL, USA	This study	

Appendix 4. Morphology characters and descriptions of states.

1. **Habit:** (0) herbaceous (incl woody at base), (1) woody,
2. **Lenticels on branches:** (0) absent, (1) present,
3. **Stipules:** (0) interpetiolar line, (1) leafy, (2) interpetiolar stipules, (3) ochrea
4. **Colleters in leaf axils:** (0) absent, (1) present
5. **Colleters inside calyx:** (0) absent, (1) present
6. **Leaf venation:** (0) eucamptodromous, (1) acrodromous,
7. **Number of bracteoles:** (0) absent, (1) 1 pair, (2) 2-3 pairs
8. **Inflorescence position:** (0) terminal, (1) axillary
9. **Inflorescence:** (0) cymose, often thyrsoid, (1) cincinnous towards branch apices, (2) solitary
10. **Flower sexuality:** (0) bisexual, (1) gynodioceous or unisexual
11. **Perianth:** (0) pentamerous, (1) tetramerous
12. **Calyx aestivation:** (0) imbricate, (1) valvate or open
13. **Calyx lobes:** (0) equal, (1) unequal, (2) calycophyllous, (3) 2-lobed foliaceous involucre
14. **Corolla aestivation:** (0) contort, (1) valvate, (2) imbricate
15. **Corolla shape:** (0) salverform, (1) rotate
16. **Corolla color at anthesis:** (0) yellow, white, green, (1) pink, red, purple
17. **Indumentum on corolla, abaxial (outside):** (0) absent, (1) present
18. **Indumentum on corolla, adaxial (inside):** (0) absent, (1) present
19. **Stamen number:** (0) same as corolla lobes, (1) one
20. **Filament length:** (0) short, (1) long
21. **Indumentum on filaments:** (0) absent. (1) present
22. **Anthers, number of cells:** (0) 2-celled, (1) 4-celled
23. **Indumentum on anthers:** (0) absent, (1) present
24. **Anther dehiscence:** (0) introrse, (1) latrorse
25. **Anther fusion:** (0) free, not connivent, (1) coherent or connivent
26. **Shape of anther apex:** (0) rounded, (1) apiculate
27. **Style:** (0) persistent, (1) deciduous
28. **Style fusion:** (0) fused or connate, (1) separate, free
29. **Indumentum on style and stigma:** (0) absent, (1) present
30. **Position and shape of placenta:** (0) axile, not stalked, (1) axile, stalked (peltate)
31. **Ovary fusion:** (0) syncarpous, (1) apically apocarpous
32. **Ovary pubescence:** (0) glabrous, (1) hairy
33. **Ovary position:** (0) superior, (1) semi-inferior
34. **Placenta:** (0) fleshy, (1) dry
35. **Mesocarp:** (0) fleshy or leathery, (1) dry
36. **Fruit dehiscence:** (0) apically dehiscent, (1) indehiscent, (2) circumsessile dehiscence
37. **Fruit walls:** (0) persistent, (1)deciduous valves with a persistent cupular base, (2) completely deciduous valves
38. **Fruit color at maturity:** (0) red, orange, (1) brown, beige, (2) white, (3) black
39. **Seed shape:** (0) polyhedral or round, (1) convex on one side, flat or concave on the other, (2) flattened

40. **Seed wing:** (0) absent, (1) present,
41. **Endosperm type:** (0) fleshy, (1) starchy, (2) horny
42. **Embryo:** (0) straight, (1) slightly curved
43. **Pollen aperture:** (0) colporate or colpate, (1) porate
44. **Pollen-polar view:** (0) angular, (1) circular
45. **Pollen ornamentation, tectum:** (0) smooth and/or perforate, (1) reticulate
46. **Scabrae on pollen:** (0) absent, (1) present
47. **Rays:** (0) exclusively uniseriate or locally biseriate, (1) uniseriate and multiseriate
48. **Vessels in tangential pairs:** (0) absent, (1) present
49. **Perforation plates with few bars:** (0) absent, (1) present
50. **Helical thickenings:** (0) absent, (1) present
51. **Fibers partly septate**: (0) absent, (1) present
52. **Fiber walls:** (0) thin, (1) thick
53. **Rays with cavities:** (0) absent, (1) present
54. **Interxylary phloem:** (0) absent, (1) present
55. **Interxylary phloem with crushed cells:** (0) absent, (1) present
56. **Seco-iridoids:** (0) absent, (1) present
57. **Indole alkaloids:** (0) absent, (1) present
58. **Cytology (x):** (0) 10, (1) 11, (2) 8

Appendix 5. Voucher information for *Strychnos* DNA extractions.

Taxa	Voucher Information: Collector name, number (voucher herb)	Collection Locality
Gardneria multiflora Makino	T. Ceming 9611186 (MO)	China
Neuburgia novocaledonica (Gilg & Benedict) J. Molina & Struwe	L. Struwe 1301 (NY)	cultivated, Hawaii
Strychnos aculeata Solered.	D. J. Harris and J. M. Fay 109 (MO)	Central African Republic
Strychnos aculeata Solered.	Merello 1338 (MO)	Ghana
Strychnos afzelii Gilg	F. N. Hepper and J. Maley 7962 (WAG)	Ivory Coast
Strychnos angolensis Gilg	F. J. Breteler 14002 (WAG)	Gabon
Strychnos angolensis Gilg	J. M. and B. Reitsma 1300 (WAG)	Gabon
Strychnos angustiflora Benth.	Hu and But 22389 (MO)	Hong Kong
Strychnos angustiflora Benth.	S. Y. Hu 8346 (MO)	Hong Kong
Strychnos arborea Hill	G. N. Bationoff 11377 (L)	Australia
Strychnos arborea Hill	K.A. Williams AQ430095 (US)	Australia
Strychnos asterantha Leeuwenb.	A. J. M. Leeuwenberg 8048 (WAG)	Ivory Coast
Strychnos asterantha Leeuwenb.	D. K. Harder et al. 2976 (WAG)	Ghana
Strychnos atlantica Krukoff & Barneby	R.P. Belem 3722 (US)	Brazil
Strychnos axillaris Colebr.	J. E. Vidal 5984 (US)	Laos
Strychnos axillaris Colebr.	J. Munzinger and F. Engelman 237 (MO)	Laos
Strychnos barteri Solered.	C. C. H. Jongkind 5002 (WAG)	Ivory Coast
Strychnos barteri Solered.	C. C. H. Jongkind et al. 1427 (WAG)	Ghana
Strychnos boonei De Wild.	A. J. M. Leeuwenberg 6593 (MO)	Cameroon
Strychnos borneensis Leenh.	M. J. E. Coode et al. 7856 (HUH)	Brunei
Strychnos brachistantha Standl.	E. Lott and S. H. Bullock 1443 (NY)	Mexico
Strychnos brasiliensis Mart.	C. Medri etal. 446 (NY)	Brazil
Strychnos campicola Gilg ex Leeuwenb.	J. Nemba et al. 615 (MO)	Cameroon
Strychnos camptoneura Gilg & Busse	F. J. Breteler 13093 (WAG)	Gabon
Strychnos chlorantha Prog.	C. Frasier 16A (CHRB)	Costa Rica
Strychnos chrysophylla Gilg	Carvalho 6197 (MO)	Guinea Ecuatorial
Strychnos cocculoides Baker	C. N. Nkhoma et al. 122 (WAG)	Zambia
Strychnos cogens Benth.	S. Mori 24607 (NY)	French Guiana
Strychnos colombiensis Krukoff & Barneby	D. Penneys 402 (MO)	Costa Rica
Strychnos congolana Gilg	L. White LJTW0872 (MO)	Gabon

Taxa	Voucher Information: Collector name, number (voucher herb)	Collection Locality
Strychnos cuminodora Leeuwenb.	C. C. H. Jongkind et al. 1525 (MO)	Ghana
Strychnos cuniculina Leeuwenb.	G. McPherson 16307 (MO)	Gabon
Strychnos dale De Wild.	A. J. M. Leeuwenberg 13569 (MO)	Gabon
Strychnos darienensis Seem.	A. Gentry 6386 (NY)	Panama
Strychnos darienensis Seem.	F. Encamacion C. 974 (MO)	Peru
Strychnos darienensis Seem.	S. McDaniel et al. 32823 (US)	Peru
Strychnos decussata (Pappe) Gilg	R. D. Noyes et al. 1031 (MO)	Madagascar
Strychnos dinklagei Gilg	A. J. M. Leeuwenberg 7932 (WAG)	Ivory Coast
Strychnos diplotricha Leeuwenb.	G. McPherson 14639 (MO)	Madagascar
Strychnos ecuadoriensis Krukoff & Barneby	D. Neill 10443 (MO)	Ecuador
Strychnos elaeocarpa Gilg ex Leeuwenb.	A. J. M. Leeuwenberg 5671 (US)	Cameroon
Strychnos erichsonii Rich. Schomb.	M.-F. Prevost and D. Sabatier 3791 (NY)	French Guiana
Strychnos eugeniaefolia Monach.	J. M. Pires et al. 50901 (US)	Brazil
Strychnos fallax Leeuwenb.	A. M. Louis 1832 (WAG)	Gabon
Strychnos fendleri Sprague & Sandwith	A. Groger 1194 (MO)	Venezuela
Strychnos floribunda Gilg	J. M. Reitsma 2020 (WAG)	Gabon
Strychnos guianensis (Aubl.) Mart.	M. Fleury 1393 (NY)	French Guiana
Strychnos henningsii Gilg	O. Pascal 266 (WAG)	Mayotte
Strychnos henningsii Gilg	P. Herman 770 (WAG)	South Africa
Strychnos hirsuta Spruce ex Benth.	D. G. Campbell et al. P22104 (MO)	Brazil
Strychnos icaja Baill.	J. Wierenga 2012 (WAG)	Cameroon
Strychnos ignatii Berg.	A. C. Church et al. 882 (HUH)	Indonesia
Strychnos ignatii Berg.	J. E. Vidal 5845 (US)	Laos
Strychnos innocua Delile	A. P. M. de Kruif 541 (WAG)	Ivory Coast
Strychnos jobertiana Baill.	R. Vasquez and N. Jaramillo 13899 (MO)	Peru
Strychnos johnstonii Hutch. & M.B. Moss	Carvalho 4213 (MO)	Guinea
Strychnos lanata A.W. Hill	A. Boonkongchart 60 (HUH)	Thailand
Strychnos lucens Baker	H. Schmidt et al. 1221 (MO)	Tanzania
Strychnos lucida R. Brown	A. Kanis 1978 (US)	Australia
Strychnos lucida R. Brown	D. D. Doejarto et al. 5846 (MO)	Thailand
Strychnos madagascariensis Poir.	G. McPherson 14555 (MO)	Madagascar
Strychnos malacoclados C.H. Wright	L. White 0712 (MO)	Gabon
Strychnos malchairii De Wild.	G. McPherson 16724 (MO)	Gabon

Taxa	Voucher Information: Collector name, number (voucher herb)	Collection Locality
Strychnos matopensis S. Moore	M. Merello et al. 970 (MO)	Zambia
Strychnos mattogrossensis S. Moore	T. B. Croat 19648A (MO)	Peru
Strychnos medeola Sagot ex Prog.	M. Fleury 1096 (NY)	French Guiana
Strychnos melinoniana Baill.	A. Henderson (305) NY	Brazil
Strychnos memecyloides S. Moore	A. J. M. Leeuwenberg 6324 (MO)	Cameroon
Strychnos millepunctata Leeuwenb.	A. J. M. Leeuwenberg 11040 (MO)	Ivory Coast
Strychnos minor Dennst.	J. Molina 23 (CHRB)	Philippines
Strychnos minor Dennst.	Ridsdale et al. ISU49 (HUH)	Philippines
Strychnos minor Dennst.	Dennis' Collectors 4601 (US)	Solomon Islands
Strychnos cf. *minor* Dennst.	J. Molina 7 (CHRB)	Philippines
Strychnos mitis S. Moore	ATBP 668 (MO)	Uganda
Strychnos mitscherlichii v. mitscherlichii Rich. Schomb.	R. Vasquez et al. 19831 (MO)	Peru
Strychnos mitscherlichii v. pubescentior Sandw.	M. J. Macia and R. Montufar 1423 (MO)	Ecuador
Strychnos mostueoides Leeuwenb.	L. Gautier and C. Chatelain LG2322 (WAG)	Madagascar
Strychnos mostueoides Leeuwenb.	S. Wolhauser SW60262 (MO)	Madagascar
Strychnos myrtoides Gilg & Busse	D. W. Thomas 3829 (MO)	Tanzania
Strychnos ngouniensis Pellegr.	A. J. M. Leeuwenberg 7885 (WAG)	Cameroon
Strychnos nicaraguensis Huft	T. B. Croat 12403 (MO)	Panama
Strychnos nigricans Prog.	M. Nee 38048 (MO)	Bolivia
Strychnos nux-blanda A.W. Hill	W. Nanakom and H. T. Beck 880200 (MO)	Thailand
Strychnos nux-vomica L.	J. F. Maxwell 90-622 (MO)	Thailand
Strychnos oiapocensis Froes	Grenand 1956 (WAG)	French Guiana
Strychnos panamensis Seem.	J. A. Duke 8604 (US)	Panama
Strychnos panamensis Seem.	M. J. Macia and H. Romero 3000 (MO)	Ecuador
Strychnos panganensis Gilg	P. Antilahimena 85 (MO)	Madagascar
Strychnos panurensis Sprague & Sandwith	F. Ayala 1556 (MO)	Peru
Strychnos parvifolia A. DC.	M. J. N. Rodal et al. 502 (MO)	Brazil
Strychnos peckii B.L. Robinson	R. Ortiz 204 (MO)	Ecuador
Strychnos pentantha Leeuwenb.	A. J. M. Leeuwenberg 13920 (MO)	Madagascar
Strychnos phaeotricha Gilg	F. J. Breteler 6481 (MO)	Gabon
Strychnos poeppigii Prog.	S. McDaniel 15267 (MO)	Peru
Strychnos polytrichantha Gilg	J. S. Burley and B. Lee 268 (HUH)	Sarawak
Strychnos potatorum L.	E. A. Banda et al. 3770 (MO)	Malawi

Taxa	Voucher Information: Collector name, number (voucher herb)	Collection Locality
Strychnos pseudoquina A. St.Hil.	H. S. Irwin et al. 13660 (MO)	Brazil
Strychnos pungens Solered.	R. R. J. van Vuuren 1842 (MO)	South Africa
Strychnos ramentifera Ducke	M. J. Macia et al. 568 (MO)	Ecuador
Strychnos rondeletioides Spruce ex Benth.	R. Vasquez 5824 (MO)	Peru
Strychnos rubiginosa A. DC.	J. R. Abbot, L. Isaacs 16406 (MO)	Bolivia
Strychnos samba Duvign.	A. J. M. Leeuwenberg 5675 (NY)	Cameroon
Strychnos scheffleri Gilg	G. McPherson 16226 (MO)	Gabon
Strychnos schultesiana Krukoff	M. F. Quigley 769 (MO)	Venezuela
Strychnos schultesiana Krukoff	R. Liesner, A. Gonzalez 9170 (MO)	Venezuela
Strychnos schunkei Krukoff & Barneby	J. Schunke 9175 (MO)	Peru
Strychnos solimöesana Krukoff	A. Gentry and S. Estensoro 70699 (MO)	Bolivia
Strychnos soubrensis Hutch. & Dalziel	M. Merello et al. 1265 (MO)	Ghana
Strychnos sp.	J. Wierenga 5140 (WAG)	Gabon
Strychnos spinosa Lam.	J. Zarucchi et al. 7614 (NY)	Madagascar
Strychnos spinosa Lam.	L. Struwe 1027 (NY)	Tanzania
Strychnos spinosa Lam.	T. Motley	cultivated, Florida
Strychnos splendens Gilg	J. Ampunsahi 1987 (MO)	Ghana
Strychnos staudtii Gilg	M. Cheek 6032 (WAG)	Cameroon
Strychnos staudtii Gilg	M. Cheek 7056 (WAG)	Cameroon
Strychnos tabascana Sprague & Sandwith	C. Frasier 20 (CHRB)	Costa Rica
Strychnos talbotiae S. Moore	P. P. C. van Meer 1296 (MO)	Nigeria
Strychnos ternata Gilg ex Leeuwenb.	A. J. M. Leeuwenberg 6231 (MO)	Cameroon
Strychnos toxifera Rob. Schomb. ex Benth.	J. S. Bissett 275 (CHRB)	Peru
Strychnos toxifera Rob. Schomb. ex Benth.	K. Thomsen 1489 (NY)	Costa Rica
Strychnos tricalysioides Hutch. & M.B. Moss	G. McPherson 16975 (MO)	Gabon
Strychnos tricalysioides Hutch. & M.B. Moss	R. M. A. P. Haegans 54 (WAG)	Gabon
Strychnos tricalysioides Hutch. & M.B. Moss	X. M. van der Burgt 560 (WAG)	Cameroon
Strychnos tseasnum Krukoff & Barneby	D. K. Evans, L. Najamdai 4408 (MO)	Ecuador
Strychnos umbellata Merill	Hu and But 22313 (MO)	China
Strychnos usambarensis Gilg ex Engl.	H. Schmidt 2206 (MO)	Ghana
Strychnos villosa A.W. Hill	J. V. LaFrankie 2387 (HUH)	Malaysia
Strychnos vitiensis A.W. Hill	A.C. Smith 6198 (NY)	Fiji
Strychnos wallichiana Steud. ex DC	A. Gentry and J. Tagi 33792 (MO)	East Malaysia

Taxa	Voucher Information: Collector name, number (voucher herb)	Collection Locality
Strychnos xantha Leeuwenb.	F. Malaisse 13311 (WAG)	Zaire
Strychnos xinguensis Krukoff	J. C. Lindeman et al. 234 (MO)	Surinam

CURRICULUM VITAE

Cynthia L. Frasier

Education

Ph.D., Plant Biology Graduate Program, Rutgers University, NJ October 2008
B.Sc., Plant Science, Cook College, Rutgers University, NJ May 2000

Academic/Teaching Experience

NSF GK-12 Teaching Fellow - Center for Mathematics, Science, & Computer Education, Rutgers University, NJ (2006-2008)

Guest speaker and mentor - Introduction to Scientific Research, Douglass College, Rutgers University, NJ (2006, 2008)

Teaching Assistant - Dept. of Plant Biology & Pathology, Rutgers University, NJ (2001-2005)

Research/Professional Experience

Postdoctoral Research Fellow – Missouri Botanical Garden, Antananarivo, Madagascar (2008)

Laboratory Manager and Chemical Hygiene Officer - Dept. of Plant Biology & Pathology, Rutgers University, NJ (2002-2008)

Curator of herbarium loans to Chrysler Herbarium (2003-2008)

Independent Research

- Training in Wood Anatomy Methodology, Royal Botanic Gardens, Kew, UK and Utrecht University, NL (Summer 2005)
- Tropical Plant Systematics, Course and field work, Organization for Tropical Studies, Costa Rica (Summer 2004)

Peer-reviewed Scientific Articles

Frasier, C. L., V. A. Albert, and L. Struwe. 2008. Amazonian lowland, white sand areas as ancestral regions of South American biodiversity: biogeographic and phylogenetic patterns in *Potalia* (Gentianaceae). Organisms, Diversity, and Evolution 8(1): 44-57.

Struwe, L., V. A. Albert, M. Fernanda Calio, **C. L. Frasier**, K. B. Lepis, K. G. Mathews, and J. R. Grant. Accepted pending revision. Evolutionary patterns in neotropical Helieae (Gentianaceae): evidence from morphology, chloroplast and nuclear DNA sequences. Taxon.

Frasier, C. L. and L. Struwe. Accepted. Phylogeny of Loganiaceae (Gentianales: Asteridae) with an emphasis on *Strychnos* using structural alignment of ITS sequences. Molecular Phylogenetics and Evolution.

Professional Contributions

Reviewer for the journals *Taxon* and *Systematic Botany*.

Provided expertise on *Strychnos* for M. Belgrano (ed.) *Catalogue of the vascular plants of the Southern Cone*.