Int. J. Plant Sci. 177(1):000–000. 2015.

1058-5893/2016/17701-00XX$15.00 DOI: 10.1086/684131

SYMPOSIUM PAPER

A MOLECULAR PHYLOGENY AND NEW INFRAGENERIC CLASSIFICATION OF *MUCUNA* ADANS. (LEGUMINOSAE-PAPILIONOIDEAE) INCLUDING INSIGHTS FROM MORPHOLOGY AND HYPOTHESES ABOUT BIOGEOGRAPHY

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Editor: Patrick S. Herendeen

Premise of research. The genus *Mucuna* has a pantropical distribution and comprises approximately 105 species, many of which show great economic value for forage, ornament, and medicine. To date, phylogenetic relationships within *Mucuna* have not been investigated using molecular data. The aim of this study was to build a phylogenetic framework for *Mucuna* to address questions about its monophyly, infrageneric relationships, divergence times, and biogeography.

Methodology. We sequenced plastid (*trn*L-F) and nuclear ribosomal (internal transcribed spacer) regions and applied Bayesian and maximum likelihood analyses. An ancestral area reconstruction coupled with a divergence time analysis was used to investigate the historical biogeography of the genus.

Pivotal results. Our results show that *Mucuna* is a monophyletic genus and that subgenus *Stizolobium* is a monophyletic group within it. We present here the analyses and results that support the need to recircumscribe subgenus *Mucuna* and to segregate a small group of species with large fruits into a newly proposed subgenus (to be described formally elsewhere after additional investigations).

Conclusions. On the basis of ancestral area reconstruction and divergence time analyses, we conclude that the genus *Mucuna* originated and first diversified in the Paleotropics around 29.2 Ma and achieved a pantropical distribution through multiple long-distance dispersal events, which were facilitated by the occurrence of seeds adapted to oceanic dispersal.

Keywords: Fabaceae, long-distance dispersal, sea-drifted seeds, systematics.

Introduction

Mucuna Adans. (Phaseoleae–Leguminosae) has a pantropical distribution and comprises approximately 105 species (Lackey 1981; Schrire 2005). The highest diversity of the genus occurs in Asia (68 taxa), followed by Oceania (34 taxa), the Americas (25 taxa), and Africa (19 taxa). Some species are widely distributed, such as *Mucuna sloanei* Fawc. & Rendle, occurring in the Americas, Hawaii, and Africa; *M. gigantea* (Willd.) DC., in Africa, Asia, and the Pacific Islands; and *M. pruriens* (L.) DC. across the entire tropical region. A number of species are ecologically and economically important, and the genus displays a high level of morphological variation, especially in its inflorescences, flowers, fruits, and seeds.

Most species of *Mucuna* are lianas (except the African endemic species *M. stans* Welw. ex Baker, which has a shrubby habit), and they are often an important component of tropical ecosystems. Because of their showy inflorescences, some species are grown as ornamentals in botanical gardens and greenhouses (e.g., *M. bennettii* F. Muell.). *Mucuna pruriens* is of wide economic importance and is currently used in agriculture as forage and green manure, for biological control, and as a coffee substitute (Duke 1981; Garcia and Fragoso 2003; Ortiz-Ceballos and Fragoso 2004; Ortiz-Ceballos et al. 2007*a*, 2007*b*). It also

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Manuscript received May 2015; revised manuscript received August 2015; electronically published November 24, 2015.

has been used for the treatment of Parkinson's disease (Nagashayana et al. 2000; Singhal et al. 2003).

Most species have pendant inflorescences; *M. stanleyi* C.T. White, endemic to New Guinea, is the only lianescent *Mucuna* with an erect inflorescence. Peduncles vary from a few centimeters long (e.g., 5–18 cm long in *M. sloanei*) to approximately 2 m long (e.g., *M. globulifera* T. M. Moura, N. Zamora & A. M. G. Azevedo). The length of peduncle is partially associated with the pollination system. Species with short-pedunculate inflorescences are mostly pollinated by birds; those with long-pedunculate inflorescences (over 1 m long) are usually pollinated by bats. The single lianescent species with an erect inflorescence is visited by and may be pollinated by a possum (H. Fortune-Hopkins, unpublished data). Inflorescences can be pseudoracemose (e.g., *M. flagellipes* Vogel, from Africa); pseudopaniculate (e.g., *M. paniculata* Baker, from Madagascar); or umbelliform, in which all flowers are closely clustered at the inflorescence apex and where the floral internodes are so reduced as to not be evident (e.g., *M. elliptica* Ruiz & Pavon, from South America). The species with pseudoracemose inflorescences are distributed across the entire geographical range of the genus; those with pseudopanicles occur in the Paleotropics, whereas all the species with umbelliform inflorescences are found in the Neotropics (i.e., *M. argentea* T. M. Moura, G. P. Lewis & A. M. G. Azevedo, *M. cajamarca* T. M. Moura, G. P. Lewis & A. M. G. Azevedo, *M. cuatrecasasii* Hern. Cam. & C. Barbosa ex. L. K. Ruiz, *M. elliptica, M. klitgaardiae* T. M. Moura, G. P. Lewis & A. M. A. Azevedo, and *M. pseudoelliptica* T. M. Moura, G. P. Lewis & A. M. G. Azevedo). Two widely distributed species (*M. sloanei* and *M. gigantea*) have a reduced pseudoraceme in which the brachyblasts and pedicels are progressively shorter toward the inflorescence apex (rather than of uniform length). This has been described as pseudoumbellate by some authors (e.g., Wilmot-Dear 1990; Tozzi et al. 2005), although the internodes are clearly visible on the inflorescence rachis.

Mucuna flowers show a remarkable variation in color of the corolla, ranging from white (e.g., *M. klitgaardiae*), cream (e.g., *Mucuna urens* (L.) Medik.), or greenish (e.g., *M. monticola* Zamora, T. M. Moura & A. M. G. Azevedo) to yellow (e.g., *M. japira* A. M. G. A. Tozzi, Agostini & Sazima), orange (e.g., *M. rostrata* Benth.), red (e.g., *M. bennetti*), purple (e.g., *M. pruriens*), or almost black (e.g., *M. hainanensis* Hayata). The wing petals of the corolla can be either longer than the standard (e.g., *M. mutisiana* (Kunth) DC.) or shorter (e.g., *M. holtonii* (Kuntze) Moldenke). The flowers vary in size from 2.5 cm long (e.g., *M. lane-poolei* Summerh.) to 11 cm long (in *M. cuatrecasasii*).

The morphology of the fruits also presents an important suite of taxonomic characters. The pod surface is sometimes ornamented by lamellae (in a transversal, longitudinal, oblique, or reticulate pattern) or ornamentation can be completely lacking. Most species have dehiscent fruits, but two (*M. poggei* Taub. and *M. occidentalis* T. M. Moura & G. P. Lewis, both endemic to Africa) have indehiscent fruits. Some species have fruits shorter than 10 cm and contain approximately five seeds (e.g., *M. pruriens*); other species have fruits 10–30 cm long, but they again contain approximately five seeds (e.g., *M. urens*); in a third group of species, the fruits can be over 50 cm long and have up to 18 seeds (e.g., *M. macrocarpa* Wall.). In addition, the seeds and hilum provide taxonomically informative characters. Seeds can be reniform, discoid, or globose; the length of the hilum varies from 3–7 mm in length (circling less than 20% of the seed circumference) to 8–9 cm in length (circling more than 50% of the seed circumference).

On the basis of fruit and seed morphology, two subgenera have been traditionally recognized in *Mucuna* (Wilmot-Dear 1984): *M.* subg. *Mucuna* and *M.* subg. *Stizolobium* (P. Browne) Baker. *Stizolobium* P. Browne was described by Browne (1756), and De Candolle (1825) later down-ranked it to a section of *Mucuna*, as *M.* sect. *Stizolobium* (P. Browne) DC. Currently, the infrageneric classification of *Mucuna* recognizes two subgenera and no sections (Wilmot-Dear 1984, 1991). Nevertheless, due to differences in fruit and seed shape and hilum length, some authors have treated *Stizolobium* as a distinct genus (e.g., Molina Rosito 1975; Stevens et al. 2001; Zamora 2010). Phylogenetic studies are necessary to clarify this issue.

Regional taxonomic studies have been published for the genus *Mucuna* across its pantropical distribution range (Verdcourt 1970, 1971, 1978, 1979*a*, 1979*b*, 1981; Wilmot-Dear 1984, 1987, 1990, 1991, 1992, 1993, 2008; Wiriadinata and Ohashi 1990; Du Puy et al. 2002; Tozzi et al. 2005; Ren and Wilmot-Dear 2010; Moura et al. 2012*a*, 2012*b*, 2013*a*, 2013*b*, 2013*c*, 2013*d*, 2013*e*, 2014, 2015; Moura and Lewis 2014; Zamora and Moura 2014), but there has been no global taxonomic survey to date. Moreover, a comprehensive phylogenetic study of *Mucuna* has never been performed. A small number of broader phylogenetic studies have included only two or three species of *Mucuna* (e.g., Kajita et al. 2001; Stefanovíc et al. 2009; Lima 2011) and thus have not adequately covered the entire geographical range or morphological variation of the genus. Although these studies have highlighted the relationships between *Mucuna* and its closest allies, the monophyly of the genus and its infrageneric groups remains to be tested. In addition, the lack of a phylogenetic framework for *Mucuna* precludes more precise inference about the area of origin of the genus and possible dispersal routes across the tropics.

In this study, we present a densely sampled phylogeny of *Mucuna* and try to answer the following taxonomic and biogeographical questions: (1) Are the genus *Mucuna* and its proposed subgenera monophyletic? (2) What are the infrageneric relationships among *Mucuna* species? (3) Do pollination systems or geographical ranges correlate to clades identified by the molecular phylogeny? (4) When and where did the genus originate, and what processes may have produced its current pantropical distribution?

Material and Methods

Taxon Sampling and DNA Extraction

Sixty-three taxa were sampled for this study, including 47 of *Mucuna* and 16 representing outgroups. Due to the wide geographical distribution of the genus, most of the samples used in this study came from herbarium collections. Material of a few species was collected in the field and stored in silica gel. Three sequences of *Mucuna* and nine outgroups were obtained from GenBank. The range of morphological variation and wide geographical distribution of *Mucuna* are represented in this study. A list of the species and specimens sampled is presented in the appendix.

For internal transcribed spacer (ITS) analysis, 53 accessions of *Mucuna* from 45 taxa were sequenced, and for the *trn*L-F region, 48 accessions of 33 taxa were sequenced. Because it was impossible to sequence all the samples for both markers due to the high level of degradation of the DNA, we opted for sequencing as much as we could for each locus and then presenting the results separately. We also present a combined analysis for the species sequenced for both markers (34 accessions, 30 taxa).

The DNA extraction from leaf tissue was conducted in three different laboratories: (1) the specimens from MO, UEC, and CEN herbaria were extracted at the Missouri Botanical Garden (St. Louis, MO) using MP FastDNA Green Spin Kit (MP Biomedicals). After extraction, the DNA was cleaned by DNA Axigen AxyPrepPCR Clean-Up Kit; (2) the specimens from K, some from L, and GH herbaria were extracted in the Jodrell Laboratory, Royal Botanic Gardens, Kew, using the 2 × cetyltrimethylammonium bromide method (Doyle and Doyle 1987), and the DNA was cleaned by a cesium chloride-ethidium bromide gradient (1.55 g/mL) and a dialysis procedure to yield material suitable for long-term storage; (3) specimens from L were extracted at Chiba University (Chiba, Japan), using the DNeasy Plant Mini Kit (Qiagen) and following the manufacturer's instructions with a modified protocol for herbarium materials. The concentration of genomic DNA was measured with a GeneQuant 100 electrophotometer (GE Healthcare, Life Sciences).

Polymerase Chain Reaction (PCR) and Sequencing

Two markers were used: the nuclear region ITS (White et al. 1990) and the plastid region *trn*L-F (Taberlet et al. 1991). When amplification of the ITS region failed, internal primers ITS2 and ITS3 (Baldwin 1992) were used to amplify the ITS region in two fragments in association with primers ITS5 and ITS4, respectively. Because the DNA obtained from the herbarium specimens is generally degraded, both regions were amplified and sequenced using internal primers for most of the samples. The PCR and sequencing steps were conducted in two laboratories: the Jodrell Laboratory, Royal Botanic Gardens, Kew, and the Department of Biology at Chiba University.

For the analysis conducted in the Jodrell Laboratory, the PCR was performed in 25-μL-volume reactions with the following components: 1.0 μL template DNA; 22.5 μL of Reddy PCR Master Mix (2.5 mM $MgCl_2$; Thermo Scientific, Waltham, MA); 0.5 μL of each primer (100 ng/μL); 5 μL of tricholse, bovine serum albumin, and tween; 1.0 μL of dimethyl sulfoxide. The same PCR mix was used for both nuclear and plastid regions. The PCR conditions for both regions were an initial denaturation at 80°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, primer annealing at 48°–50°C for 1 min, and primer extension at 65°C for 1 min; this was followed by a final extension step of 7 min at 64°C. For some samples that were difficult to amplify, the PCR conditions included a ramp of 0.3°C/s, as described by Shaw et al. (2007).

PCR products were checked on 1% agarose gel before being cleaned with QIAquick PCR purification kit (Qiagen). Cycle sequencing reactions were performed in 5-μL-volume reactions, using 0.3–1.0 μL of the PCR product, 0.25 μL BigDye, 1.5 μL BigDye Buffer, 1.5 μL double distilled water (ddH_2O), and 0.75 μL of the same primer as for PCR (diluted to 10%). The cycle sequencing products were cleaned using Magnesil and the automated workstation BiomeK NX58 (Beckman Coulter). Complementary strands were sequenced on an ABI 3730 automated sequencer (Applied Biosystems) and then assembled; software base-calling was verified using Sequencher 4.5 (Gene Codes, Ann Arbor, MI).

At Chiba University, PCR reactions were performed in volumes of 10 μL containing 0.2 units of ExTaq (TaKaRa) or 0.25 units of MightyAmp DNA Polymerase (TaKaRa) and 0.2 mM deoxynucleotide triphosphates, 10 × PCR buffer containing 1.5 mM magnesium chloride, 0.5–1 μM of each primer, and 20 ng of genomic DNA. The PCR conditions were as follows: 2 min for initial denaturation at 95°C, followed by 35 amplification cycles of 45 s denaturation at 95°C, 1 min annealing at 56°C, 1 min extension at 72°C, and a final 10 min extension at 72°C. The PCR products were visualized on a 0.8% agarose gel. PCR products were purified using illustra ExoStar Enzymatic PCR and Sequencing Clean-Up Kit (GE Healthcare) according to the manufacturer's instructions. The cycle sequencing reactions were performed using the BigDye Terminator, version 3.1, Cycle Sequencing Kit (Applied Biosystems), and cycle sequencing products were purified using an ethanol precipitation method. All base sequences were determined using an ABI 3500 DNA sequencer (Applied Biosystems).

Phylogenetic Analyses

The sequences for all DNA regions were assembled into contigs and edited using the program Geneious, version 7.1.7 (Biomatters, Aukland, New Zealand), or SeqScape, version 2.7 (Life Technologies, Applied Biosystems). A BLAST search (http://blast.ncbi.nlm.nih.gov/) was conducted for all sequences to check for possible contaminant DNA. Afterward, edited alignments were performed using the Clustal W (Larkin et al. 2007) and MUSCLE (Edgar 2004) programs using default settings with manual adjustments.

We performed phylogenetic analyses using two different approaches: Bayesian inference (BI) and maximum likelihood (ML). The Bayesian analysis was performed using a Markov chain Monte Carlo (MCMC) method, as implemented in MrBayes, version 3.2.2 (Ronquist et al. 2012). The best-fit model of DNA substitution for each molecular region was determined using MrModeltest, version 2.2 (Nylander 2004), and the Akaike information criterion (Akaike 1974). The GTR + I + G and GTR + G models were selected as the "best model" for the ITS and *trn*L-F regions, respectively. For the combined analysis, two partitions were defined corresponding to the plastid and nuclear regions. Two independent Metropolis-coupled MCMCs with incremental heating temperature of 0.25 were run for 50 million generations, with the parameters and the resulting phylogenetic trees being sampled every ten-thousandth generation. The analysis was repeated four times. The MCMC sampling was considered sufficient when the effective sampling size (ESS) for each parameter was higher than 200, as verified with Tracer, version 1.6 (Rambaut et al. 2014). A burn-in period of one million generations per run was applied, and the remaining trees were used to reconstruct an "allcompat" consensus tree with posterior probabilities (PP) for each node. Members of tribe

Desmodieae, as well as *Apios americana* Medik., were selected as outgroup taxa based on published phylogenies (Kajita et al. 2001; Schrire 2005; Stefanovíc et al. 2009).

The ML analysis was performed using Randomized Axelerated Maximum Likelihood (RAxML), version 8.1.11 (Stamatakis 2014), which implements a rapid hill-climbing algorithm (Stamatakis 2006). Analyses were run for the best-scoring ML tree inferences under the GTR-GAMMA model. Rapid bootstrapping was performed with 1000 replications using the GTR-CAT estimation to assess branch support (Stamatakis 2006). To increase analysis speed, parallel versions of the RAXML, MPI/Pthreads were used (Pfeiffer and Stamatakis 2010).

Partition Homogeneity Test

To assess congruence between the *trn*L-F and ITS data sets, we performed the incongruence length difference (ILD) test (Farris et al. 1994), implemented as a partition homogeneity test in PAUP*, version 4.0b10 (Swofford 2002). The test was conducted using a heuristic search with tree-bisection-reconnection branch-swapping algorithm and with invariant characters excluded (Cunningham 1997). Three random additions per replicate with a time limit of 10 min were selected to run 1000 homogeneity replicates.

Divergence Time Analysis

Estimates of divergence time were obtained using the Bayesian inference approach implemented in the package Bayesian Evolutionary Analysis Sampling Trees (BEAST), version 1.8.1 (Drummond et al. 2012), using the combined matrix of ITS and *trn*L-F, applying the same partition delimitation and evolutionary models as those used for the MrBayes analysis. We used an uncorrelated relaxed molecular clock with a lognormal distribution of rates and a Yule speciation model (Yule 1925; Gernhard 2008). The analysis was run for 30 million generations, sampling one tree every one-thousandth generation. As a calibration point, we applied a normal prior distribution (mean ± standard deviation = 39.7 ± 2.0 Ma) to the root of the tree, based on the age estimate of the most recent common ancestor of *Platycyamus regnellii* Benth. and *Phaseolus vulgaris* L., published in Lavin et al. (2005). To summarize plausible trees and to obtain a maximum clade credibility tree, the Tree Annotator program implemented in the BEAST package was used. Twenty-five percent of trees (i.e., 7500 trees) were excluded as burn-in from the subsequent calculations. Tracer, version 1.6 (Rambaut et al. 2014), was used to check the ESSs, convergence, and confidence intervals (CIs). The trees were visualized and edited using FigTree, version 1.4.2.

Biogeographic Inferences

To investigate the historical biogeography of *Mucuna*, we conducted ancestral state geographic distributions on phylogenetic trees using the Bayesian Binary MCMC (BBM) method implemented in the Reconstruct Ancestral State in Phylogenies (RASP) program, version 3.2 (Yu et al. 2015). We divided the pantropical distribution of *Mucuna* into eight areas that were based on the presence of endemic species: North America, including Mexico (A), Asia (B), Central America (C), Papua New Guinea (D), South America (E), Africa (F), Pacific (G), and Madagascar (H). BBM calculates the probabilities of ancestral ranges using the probabilities for each unit area. A condensed tree created by Tree Annotator from the output of the BEAST analysis on the basis of a combined data set of *trn*L-F and ITS was used. MCMC calculations were conducted with 2,000,000 generations and a sample frequency of 1000. We used a FixedJC (Jukes-Cantor) model with the number of chains equal to 10 and excluding 200 samples as burn-in using null root distribution. The maximum number of areas selected was six.

Results

Phylogenetic Analyses

The aligned *trn*L-F matrix consisted of 1203 characters for 58 samples, including 48 of *Mucuna* (33 taxa) and 10 outgroup taxa; the aligned ITS matrix consisted of 902 characters for 62 samples, 53 of *Mucuna* (45 taxa), and nine outgroup taxa. For both markers, *Mucuna* was supported as monophyletic (figs. 4, 5). For the *trn*L-F marker, both subgenera traditionally recognized within *Mucuna* were also monophyletic (fig. 4), whereas *Mucuna* subg. *Mucuna* appeared as nonmonophyletic in the analysis based on the ITS marker (fig. 5). A third clade, here named the Macrocarpa clade, was revealed.

The ILD analysis suggested that the *trn*L-F and ITS data sets are incongruent ($P < 0.003$). This incongruence was also detected from visual inspection of trees derived from individual independent analysis of the plastid and nuclear regions (figs. 4, 5). The incongruence observed is mainly related to the position of the Macrocarpa clade; otherwise, no incongruence was found and the topologies of the main clades from each region were congruent with support >0.7 posterior probability and >70% bootstrap. In the *trn*L-F tree, the Macrocarpa clade (*M. birdwoodiana, M. calophylla, M. macrocarpa,* and *M. sempervirens*) is sister to the core Mucuna clade (fig. 5), whereas in the ITS trees (fig. 4) and combined trees (fig. 1), it is sister to the Stizolobium clade. Given that some authors argue that combining different data sets (fig. 1) generally improves phylogenetic accuracy, to increase the resolution of our trees regardless of their incongruence (Cunningham 1997; Yoder et al. 2001), we decided to merge data sets and perform a combined analysis.

The majority rule consensus tree resulting from the Bayesian analysis of the combined data set revealed that the genus *Mucuna* is monophyletic (fig. 1). Three main clades were resolved, here named the core Mucuna clade (which includes the type species of *Mucuna*, *M. urens*), which thus represents *Mucuna* subg. *Mucuna*; the Stizolobium clade (which includes the species currently placed in *M.* subg. *Stizolobium*); and the Macrocarpa clade. The main diagnostic characteristic of species in the Macrocarpa clade is the long pods, and therefore Macrocarpa is an appropriate name for the clade (T. M. Moura, unpublished data). The results obtained from the ML analysis are in agreement with the Bayesian results (results not shown).

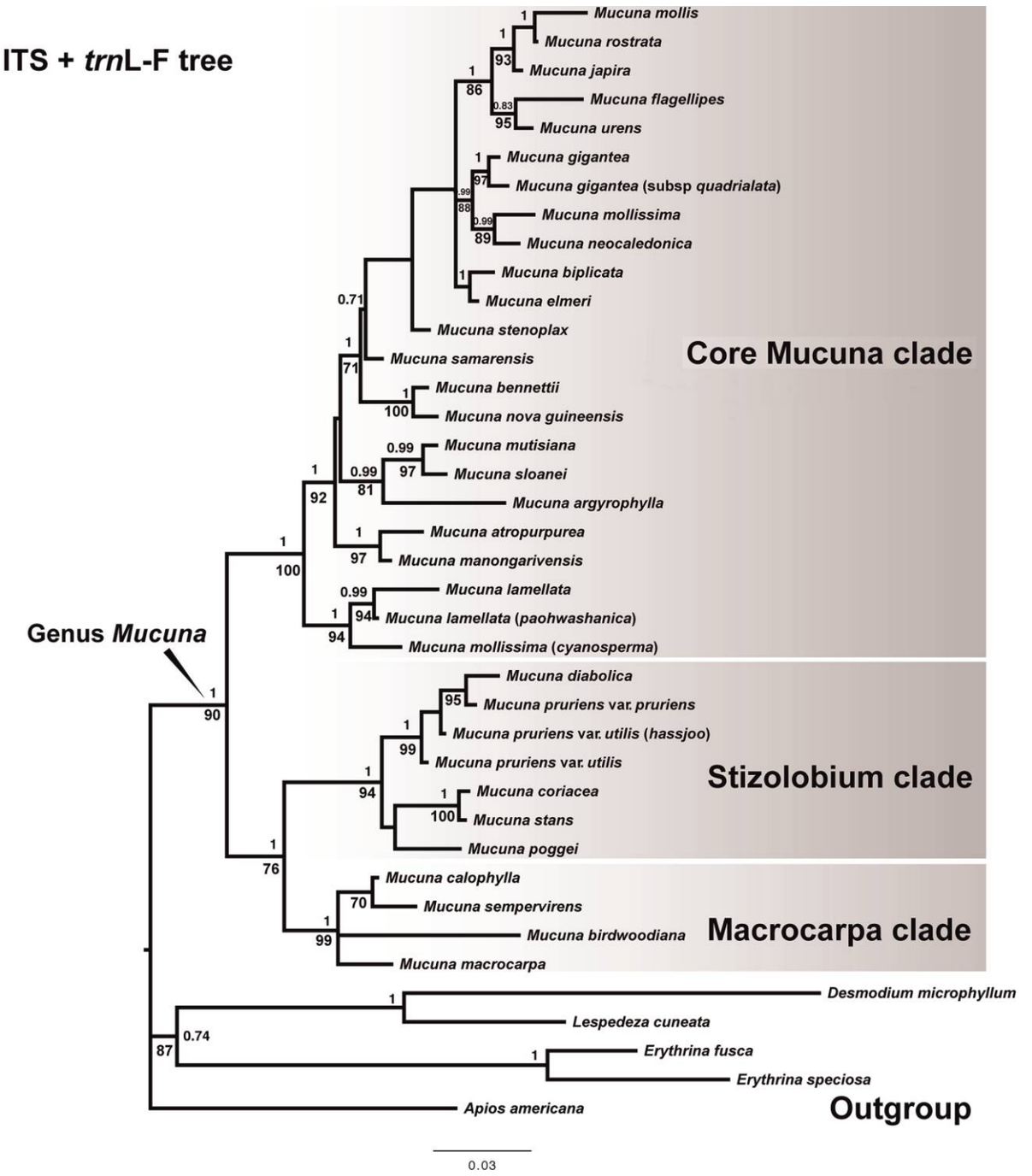

Fig. 1 Fifty percent majority rule consensus tree resulting from Bayesian analysis of the combined data set of *trn*L-F and internal transcribed spacer (ITS) sequences for *Mucuna* species. The numbers above and below branches are posterior probabilities and bootstrap supports, respectively. Values <0.7 and <70% are not shown.

Estimates of Divergence Times

The BEAST analysis based on the combined data set (fig. 2) estimated the stem age of *Mucuna* to be in the Oligocene to early Miocene (29.2 Ma; 95% CI, 18.1–39.1). The subgenus *Mucuna* (Mucuna clade) and the clade comprising the Stizolobium and Macrocarpa subclades began diversifying during the Miocene at 20.8 Ma (95% CI, 11.4–31.0) and 20.8 Ma (95% CI, 10.5–32.1), respectively. Additionally, diversification of subgenus *Stizolobium* (Stizolobium clade) and the Mac-

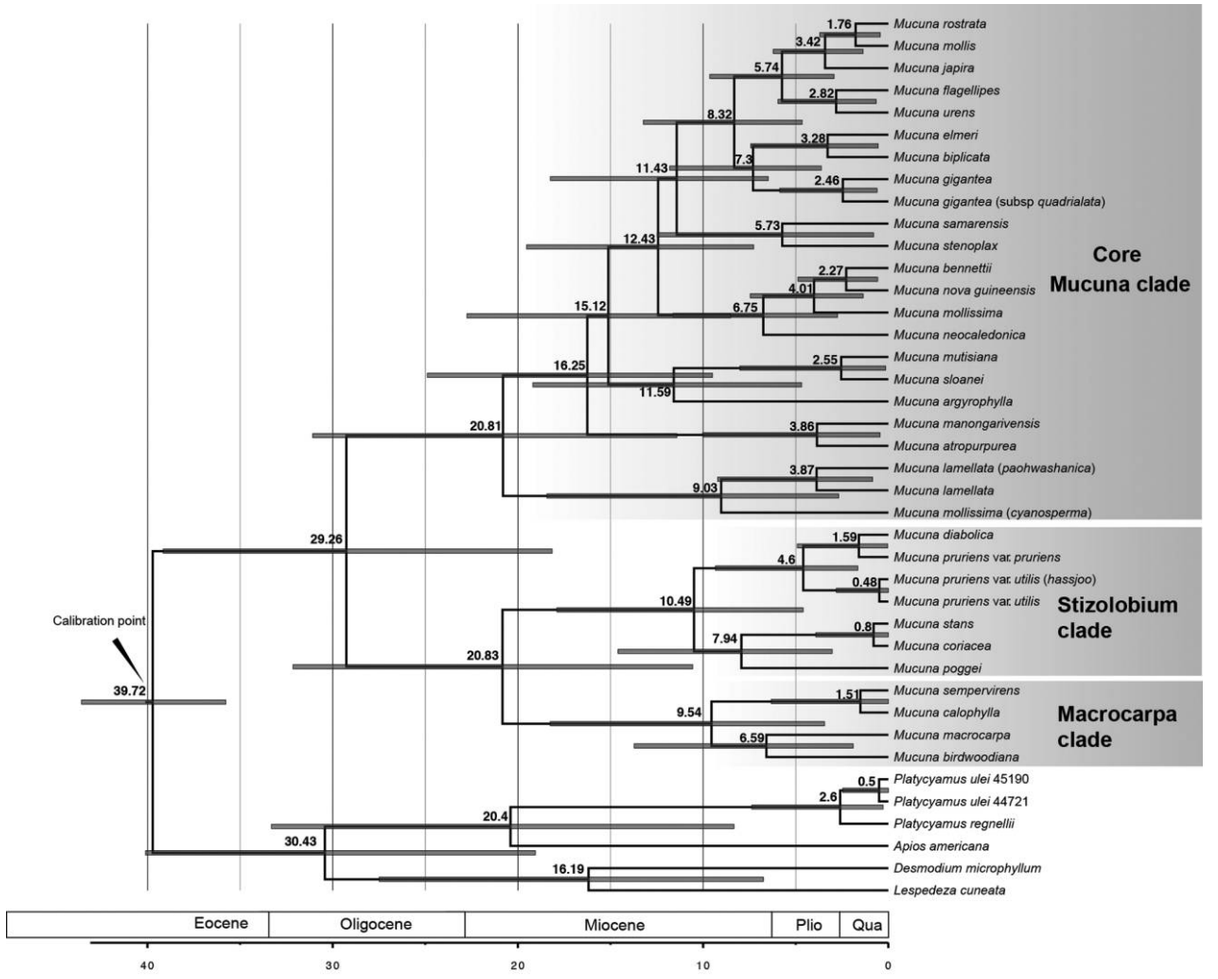

Fig. 2 Chronogram for the genus *Mucuna*, based on the concatenated matrices of *trn*L-F and internal transcribed spacer markers obtained with a BEAST analysis. The numbers at nodes refer to mean age of the nodes (Ma). Gray bars represent 95% confidence intervals for the estimated mean dates. Qua: Quaternary, Plio: Pliocene.

rocarpa clade occurred in the late to middle Miocene around 10.4 Ma (95% CI, 4.5–17.8) and 9.5 Ma (95% CI, 3.4–18.2), respectively (fig. 2). Our results suggest that the main diversification of the genus *Mucuna* occurred more recently, in the middle to late Miocene and Pliocene.

Biogeographic Inferences

The ancestral geographic ranges obtained by BBM analysis (fig. 3) suggest that *Mucuna* originated in area B (Asia, node I) with a marginal probability (MP) of 91.5%. Subclade *Mucuna* (node II) and the subclade comprising the Macrocarpa and Stizolobium clades (node III) are also postulated to have an Asian origin with MPs of 95.9% and 85.4%, respectively. Although the Macrocarpa clade has an exclusively Asian origin (node IV, MP = 97.6%), the Stizolobium clade (node V) is ambiguous, and five ancestral areas are possible, B (MP = 37.7%), BF (MP = 20%), AB (MP = 7%), F (MP = 6.3%), and BE (MP = 5.7%), with 23% ambiguity (fig. 3, node V). Node VI was inferred to be of South American (area E) origin with MP = 73.5%, suggesting a dispersal event from Asia to the Neotropics.

Discussion

Phylogenetic Analyses

Mucuna was recovered as monophyletic in all analyses and comprises three main clades, two of them corresponding to *M.* subg. *Mucuna* as traditionally circumscribed. However, the position of the Macrocarpa clade varies between the analyses, suggesting that additional molecular data, coupled with a detailed morphological analysis, are needed to clarify this relationship.

The Macrocarpa clade is characterized by species with long fruits (often >50 cm in length, containing up to 18 seeds), whereas the remaining species in the core Mucuna clade (i.e., excluding the Macrocarpa clade) have fruits up to 30 cm long that usually contain no more than five seeds. Members of the

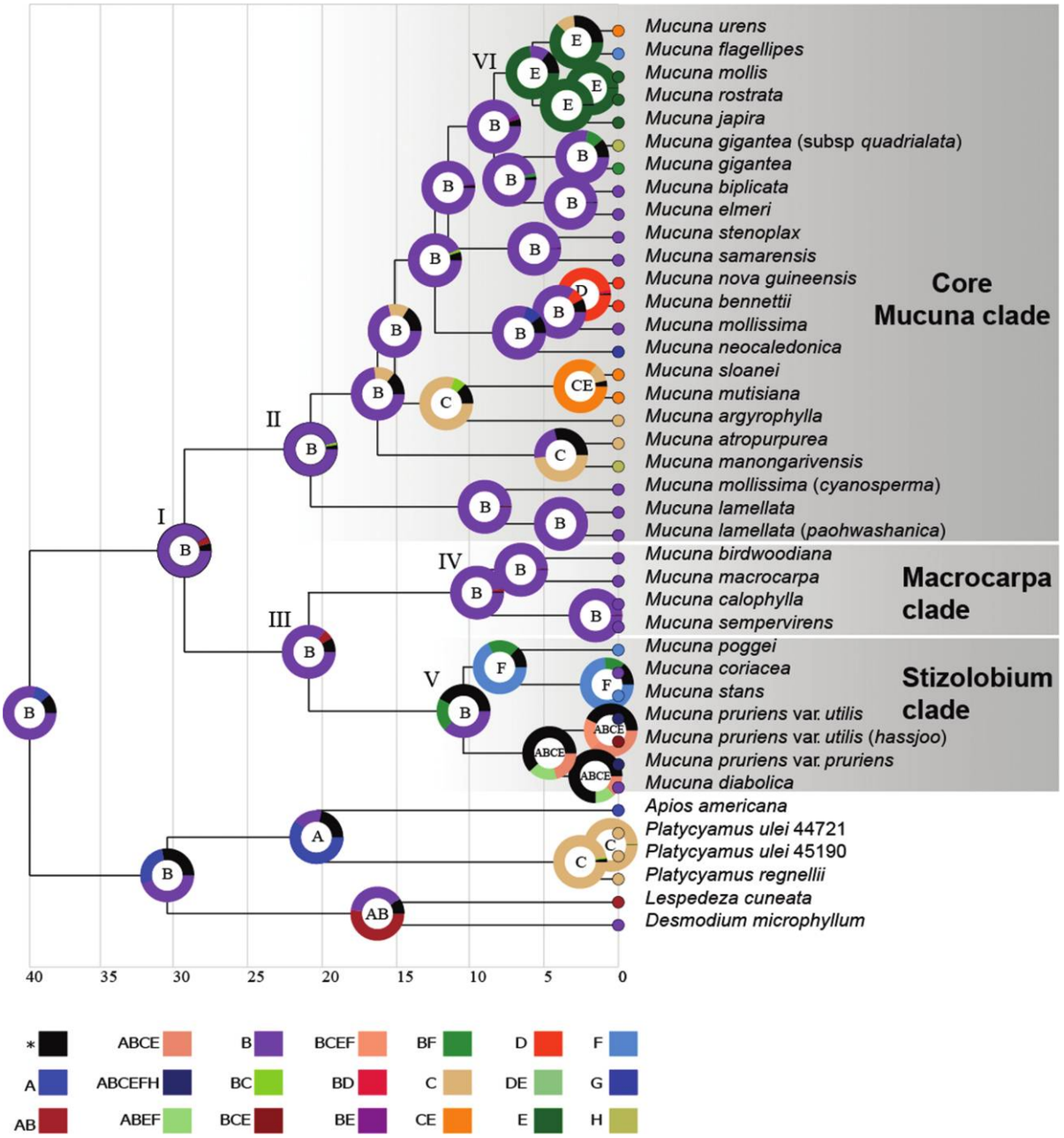

Fig. 3 Output of the Bayesian binary Markov chain Monte Carlo analysis from the RASP program, version 3.2 (Yu et al. 2015), showing ancestral geographic reconstructions at each node of the phylogenetic tree of *Mucuna*. The proportion of colors in circles at each node represents possible ancestral ranges, and letters in the centers of the circles show the most likely state of each node. Numbers on the horizontal axis correspond to the estimated ages (Ma) obtained from the BEAST analysis. A = North America, B = Asia, C = Central America, D = Papua New Guinea, E = South America, F = Africa, G = Pacific, and H = Madagascar. Black represents other ancestral ranges.

Stizolobium clade (*M.* subg. *Stizolobium*) also have fruits up to 10 cm long that usually contain no more than five seeds, but the seeds are reniform in outline (rather than round, as they are in the other two clades), and the hilum is shorter. Although all the taxa of subgenus *Stizolobium* cluster in a well-supported clade, the topology of the trees indicates that this group should be treated as a subgenus within *Mucuna* (Wilmot-Dear 1984, 1991; Moura et al. 2013*b*, 2014), instead of as an independent genus, as suggested by some authors (Molina Rosito 1975; Stevens et al. 2001; Zamora 2010).

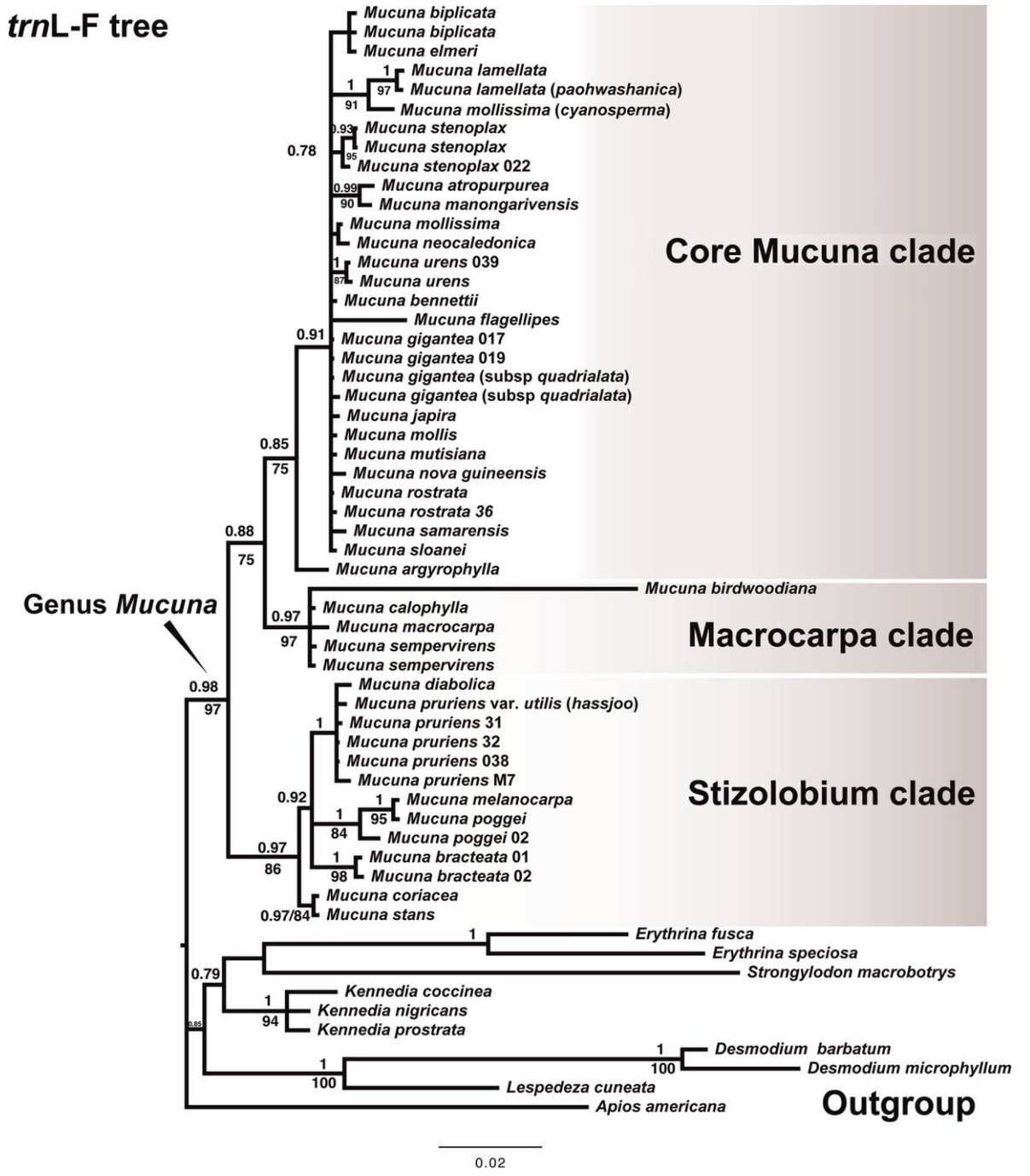

Fig. 4 Fifty percent majority rule consensus tree resulting from Bayesian analysis of the *trn*L-F marker for *Mucuna* species. The numbers above and below branches are posterior probabilities and bootstrap supports, respectively. Values <0.7 and <70% are not shown.

As stated above, the Macrocarpa clade is morphologically coherent with respect to fruit morphology. Only the ML analysis of the ITS marker failed to include *M. calophylla* as a member of the Macrocarpa clade, placing it instead as the sister taxon to subgenus *Stizolobium*, although with low support (58%). Conversely, there is high support (99% bootstrap and 1 posterior probability support) in the combined ML and BI analyses for a clade comprising *M. birdwoodiana*, *M. calophylla*, *M. macrocarpa*, and *M. sempervirens*. Incongruency between the chloroplast DNA and ITS trees regarding the position of the

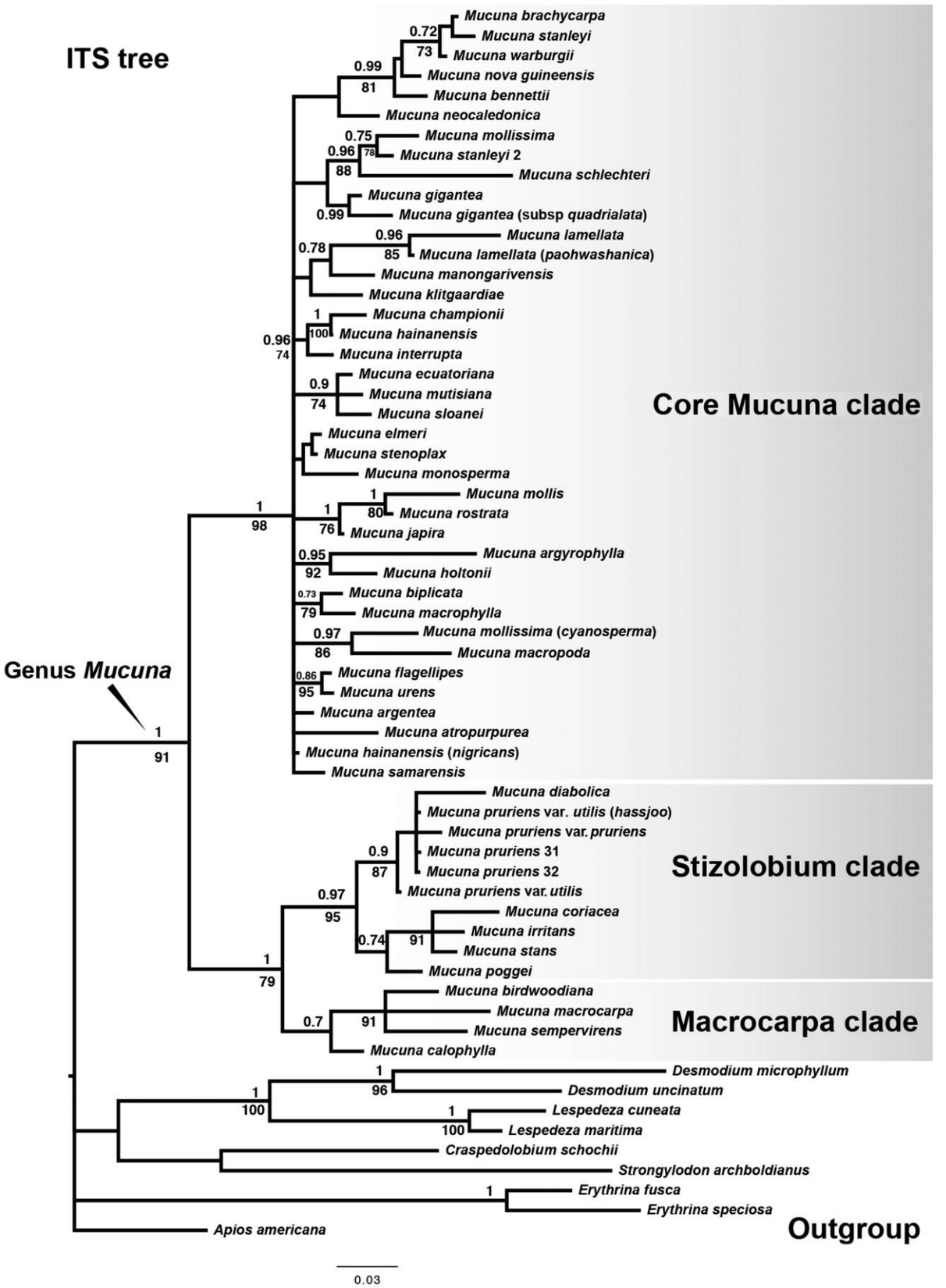

Fig. 5 Fifty percent majority rule consensus tree resulting from Bayesian analysis of the internal transcribed spacer (ITS) marker for *Mucuna* species. The numbers above and below branches are posterior probabilities and bootstrap supports, respectively. Values <0.7 and <70% are not shown.

Macrocarpa clade (see above) and the observed variation in polyploidy level among individuals of *M. sempervirens* might be the result of incomplete lineage sorting and/or introgressive hybridization (Maddison and Knowles 2006; Petit and Excoffier 2009) among species of the Macrocarpa, core Mucuna, and Stizolobium clades.

The corolla color, the pollination systems, and the lamellate ornamentation of the pod surface were not found to be synapomorphies for any of the three major clades in the analyses. For example, *M. nova-guineensis* and *M. bennettii*, two red-flowered species from Oceania, are placed in the same subclade, whereas *M. neocaledonica* (from New Caledonia) and *M. rostrata* (Neotropics), which have red, purplish-red, or orange flowers, are nested in different subclades. Similarly, *M. japira* (endemic to Brazil) and *M. sloanei* (widely distributed) both have yellow flowers but are not closely related. There is also no evident clustering of species with similar pollination systems. Species bearing long peduncles, which are characteristic of bat pollination, appear in different subclades. For example, in the clade formed by *M. urens, M. flagellipes, M. mollis, M. rostrata*, and *M. japira*, the first three species have long peduncles and are bat pollinated, whereas the other two species have short peduncles and are pollinated by birds. Likewise, in the clade comprising *M. argyrophylla, M. mutisiana*, and *M. sloanei*, the first two species have long peduncles and are probably bat pollinated, whereas *M. sloanei* has a short peduncle and is likely to be bird pollinated. Our results suggest that the pollination syndrome and floral features have arisen in parallel multiple times during the evolutionary history of *Mucuna*, a trend that has been reported in other plant groups, such as Sinningeae (Gesneriaceae; Perret et al. 2003) and Bignonieae (Bignoniaceae; Alcantara and Lohmann 2010). The presence of lamellate ornamentation on the pod surface is apparently not synapomorphic (e.g., *M. argyrophylla, M. mollis*, and *M. gigantea*, which lack ornamented pods, group into different subclades, together with species with ornamented fruit). No clustering of species on the basis of inflorescence type, namely, pseudoracemose, pseudopaniculate, or pseudoumbellate, was observed, although the inclusion of several Neotropical pseudoumbellate species (*M. argentea, M. cajamarca, M. cuatrecasasii, M. elliptica, M. klitgaardiae*, and *M. pseudoelliptica*), which were not included in our study, would be needed to further support this finding. On the other hand, the types of fruit, seed, and seed hilum do provide taxonomically informative characters that support our molecular findings.

Biogeographic Inferences

Present-day global legume distributions are most likely a combination of long-distance dispersal (LDD) and vicariance, based on the evidence from phylogenetic studies and fossil records (Schrire et al. 2005; Bessega et al. 2006). More importantly, the transoceanic distribution of various crown clades of the legumes is considered to be the result of LDD, because the young ages estimated for most legume groups preclude vicariance as an explanation for their disjunct distributions (Lavin et al. 2004). According to our divergence time analysis using BEAST, the genus *Mucuna* evolved sometime in the Oligocene to early Miocene (39.1–18.1 Ma), whereas the core Mucuna, Macrocarpa, and Stizolobium clades diversified in the Miocene (fig. 2). Our results suggest that the genus *Mucuna* originated in Asia and has since undergone multiple colonization events into Africa and North, Central, and South America. Although the role of long-distance dispersal in the biogeography of land plants has generally been underestimated (Cain et al. 2000; Vatanparast 2010), when one considers the topology of our phylogenetic trees and ancestral area reconstruction, it is plausible that several LDD events underpin the pantropical distribution of *Mucuna* across Asia, Africa, and the Americas. The majority of extant *Mucuna* species (>80%) are restricted to a single continent, whereas some species, *M. gigantea* and *M. sloanei*, have almost pantropical distributions. The distribution of these species is hypothesized to be a result of dispersal of seeds that have drifted by ocean currents, because *Mucuna* seeds have been found even along the coast of New Zealand (Mason 1961) and on beaches in Europe (Nelson et al. 2000). LDD plays an important role in the biodiversity and biogeography of a number of legume genera, including *Apios* (Li et al. 2014), *Canavalia* and *Dalbergia* (Vatanparast et al. 2011; Vatanparast et al. 2013), *Lonchocarpus* (Silva et al. 2012), *Zornia* (Fortuna-Perez et al. 2013), and members of tribe Fabeae (Schaefer et al. 2012) and indeed of legumes in general (Lavin et al. 2004; Pennington et al. 2006).

Although our taxon sampling could be increased, our analyses showed that at least two dispersal events from the Paleotropics (Asia) to the Neotropics can be inferred. One dispersal event to Central America has led to a clade comprising *M. sloanei, M. mutisiana*, and *M. argyrophylla*, whereas another inferred dispersal event to South America has resulted in a clade comprising *M. urens, M. mollis, M. rostrata*, and *M. japira*. For Africa, four dispersal events can be inferred. Although the African species of *M.* subg. *Stizolobium* (*M. coriacea, M. poggei, M. pruriens*, and *M. stans*) could have resulted from a single colonization from Asia, *M.* subg. *Mucuna* arrived on the African continent multiple times: *M. flagellipes* from South America, *M. gigantea* from Asia, and *M. sloanei* from Central America (all by LDD). *Mucuna manogarivensis*, which is endemic to Madagascar, is sister to *M. atropurpurea* from Asia, but the ancestor of these two species appears to be Central American (although this relationship received low support of PP; 0.53). The species from Oceania, *M. nova-guineensis, M. bennettii*, and *M. mollissima*, also have an origin in Asia (fig. 3), whereas the origin of *M. diabolica* (an Australian species) is uncertain.

Conclusions

Our results confirm that the genus *Mucuna* is monophyletic, as is the previously described subgenus *Stizolobium*. *Mucuna* subg. *Mucuna* contains two main clades, the core Mucuna clade and Macrocarpa clade. Although the position of the Macrocarpa clade is variable in our analyses, it is consistent in species content for both studied markers. The species of this clade are morphologically distinctive with respect to fruit type, but we are carrying out additional investigations before formally describing a new subgenus. On the basis of the divergence time analysis, we conclude that the genus *Mucuna* originated in the Paleotropics, in the Oligocene to early Miocene, and gradually expanded its geographic range via a number of dispersal events into

Africa and via at least two dispersal events into the Neotropics. These findings suggest that long-distance oceanic dispersal of *Mucuna* seeds has had a central role in forming the present-day pantropical distribution of and diversification within the genus.

Acknowledgments

We thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, and the Science without Borders Program for the postdoctoral scholarship to T. M. Moura (process 245590/2012-9); Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil (process 2012/04635-8), and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Brazil (process E-26/110.331/2012), for financial support; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil, for T. M. Moura's PhD scholarship; the Shirley A. Graham Fellowship, Missouri Botanical Garden, to T. M. Moura; the Biologia Vegetal postgraduate program at Universidade Estadual de Campinas (UNICAMP), Brazil; the Royal Botanic Gardens, Kew; and the Missouri Botanical Garden for support. We also thank Dra. Anete Pereira de Souza and Centro de Biologia Molecular e Engenharia Genética (CBMEG) Laboratory, Brazil, for support at the beginning of this study; the Jodrell Laboratory staff (Royal Botanic Gardens, Kew), especially Edith Kapinos, Dion Devey, László Csiba, Jim Clarkson, and Tim Fulcher, for valuable technical support; we thank the curators of the herbaria at K, L, and MO for providing DNA material; all researchers and friends that helped with laboratory activities, including DNA extraction and data analysis, particularly Ana Paula Fortuna Perez, André Simões, Anne Bruneau, David Bogler, Domingos Cardoso, Eve Lucas, Fiorella Fernanda Mazine Capelo, Helen Fortune-Hopkins, Laura Lima, Marcos José da Silva, Mariana de Oliveria Bünger, and Valentina D'Amico. M. Vatanparast thanks Tetsukazu Yahara and Shuichiro Tagane from Kyushu University for providing fresh material of *Mucuna*. M. Vatanparast and T. Kajita were supported by a grant from the Environment Research and Technology Development Fund (S-9) of the Ministry of the Environment, Japan. We thank Dr. Benjamin Torke and one anonymous reviewer for constructive comments on the first submitted draft of the paper and also Patrick Herendeen for his valuable editorial input.

Appendix

Voucher Information and GenBank Numbers

Shown below are voucher information and GenBank numbers (*trn*L-F and ITS) for all specimens used in this study.

Outgroups

Apios americana Medik., *ITS*: AF467019.1, *trn*L-F: EU717312.1. *Craspedolobium schochii* Harms, China, *A. Henry 9241-A* (K), ITS: KT696028. *Desmodium barbatum* (L.) Benth., *trn*L-F: EU717290.1. *Desmodium microphyllum* (Thunb.) DC., *ITS*: GQ413945.1, *trn*L-F: JN402868.1. *Desmodium uncinatum* (Jacq.) DC., *ITS*: GQ413950.1. *Erythrina fusca* Lour., Brazil: cultivated in Rio de Janeiro Botanical Gardens, ITS: KT729507; *trn*L-F: KT729512. *Erythrina speciosa* Andrews, Brazil, *T. M. Moura 1004* (UEC), ITS: KT729508; *trn*L-F: KT729513. *Kennedia coccinea* (Curtis) Vent., Australia, *T. R. Lally 1568* (MO), *trn*L-F: KT696082. *Kennedia nigricans* Lindl., cultivated in the United States, *H. Van der Werff 8254* (MO), *trn*L-F: KT696083. *Kennedia prostrata* R. Br., Australia, *R. J. Smith & A. Shade 39* (K), *trn*L-F: KT696084. *Lespedeza cuneata* (Dum. Cours.) G. Don, ITS: GU572175.1, *trn*L-F: JN402793.1. *Lespedeza maritima* Nakai, ITS: GU572190.1. *Strongylodon archboldianus* Merr. & L. M. Perry, New Guinea, *M. Fallen 404* (MO), ITS: KT696078. *Strongylodon macrobotrys* A. Gray, Brazil: cultivated in Rio de Janeiro Botanical Gardens, *trn*L-F: KT696131.

Platycyamus:

Platycyamus regnellii Benth., Brazil, *B. A. S. Pereira & D. Alvarenga 2474* (K), KT696079. *Platycyamus ulei* Harms 44721, Brazil, *E. Ule 9496* (K), *trn*L-F: KT696080. *Platycyamus ulei* Harms 45190, Peru, *E. Meneces s.n.* (MO 4268531), *trn*L-F: KT696081.

Mucuna:

Mucuna argyrophylla Standl., Mexico, *M. Sousa 11399* (MO), ITS: KT696029, *trn*L-F: KT696085. *M. argentea* T. M. Moura, G.P. Lewis & A.M.G. Azevedo., Colombia, *B. Kats & A. van Dulmen AVD 265* (K), ITS: KT729509. *M. atropurpurea* (Roxb.) DC., Ceylon, *A. G. Robyns 7327* (K), ITS: KT696030, *trn*L-F: KT696086. *M. bennettii* F. Muell., Brazil, cultivated in the Rio de Janeiro Botanical Garden, *T. M. Moura 996* (UEC), ITS: KT696031, *trn*L-F: KT696087. *M. biplicata* Teijsm & Binn., Malaysia, *L.G. Saw* (L 0462392), *trn*L-F: KT696088. *M. biplicata* Teijsm & Binn., Malaysia, *C. Hansen 136* (K), ITS: KT696032, *trn*L-F: KT696089. *M. birdwoodiana* Tutcher, China, *W. T. Tang 20607* (MO), ITS: KT696033, *trn*L-F: KT729514. *M. brachycarpa* Rech., Fiji, *W. Greenwood 1109* (K), ITS: KT696034. *M. bracteata* DC. ex Kurz, Thailand, *E. F. Anderson 4108* (MO), *trn*L-F: KT696090. *M. bracteata* DC. ex Kurz 02, Burma, *G. B. Vogt 495* (K), *trn*L-F: KT696091. *M. calophylla* W. W. Sm., China, *R. C. Ching 21690* (GH), ITS: KT696035, *trn*L-F: KT696092. *M. championii* Benth., Hong Kong, *Hu & But 20316* (MO),

ITS: KT696036. *M. coriacea* Baker, Zimbabwe, *R. D. Barnes s.n.* (K), ITS: KT696037, *trn*L-F: KT696093. *M. diabolica* Backer ex Keuch., Australia, *K. Kenneally 6391* (K), ITS: KT696038, *trn*L-F: KT696094. *M. ecuatoriana* T. M. Moura, G.P. Lewis & A.M.G. Azevedo., Ecuador, *M. Blanco 2532* (MO), ITS: KT696039. *M. elmeri* Merr., Indonesia, *Ambriansyah* (L 0501638), ITS: KT696040, *trn*L-F: KT696095. *M. flagellipes* Vogel ex Benth., Ghana, *C. C. H. Jonkind & D. K. Abbin 1588* (MO), ITS: KT696041, *trn*L-F: KT696096. *M. gigantea* (Willd.) DC., Philippines, *C. E. Ridsdale* (L 0460611), ITS: KT729510, *trn*L-F: KT696097. *M. gigantea* (Willd.) DC. 19, Hawaii, *G. Spence s.n.* (MO), *trn*L-F: KT696098. *M. gigantea* (Willd.) DC. [as *M. gigantea* subsp. *quadrialata* (Baker) Verdc.], Kenya, *S. H. Robertson & Q. Luke 5438* (MO), *trn*L-F: KT696099. *M. gigantea* (Willd.) DC. [as *M. gigantea* subsp. *quadrialata* (Baker) Verdc.], Kenya, *S.A. Robertson & Q. Luke* (72675MO), ITS: KT696042,*trn*L-F: KT696100. *M. hainanensis* Hayata, ITS: GU217596.1. *M. hainanensis* Hayata (as *M. nigricans* (Lour.) Steud.), Taiwan, *W. L. Wagner 6735* (K), ITS: KT696043. *M. holtonii* (Kuntze) Moldenke, Colombia, *W. Devia et al. 2302* (MO), ITS: KT696044. *M. interrupta* Gagnep., ITS: AB775135.1. *M. irritans* Burtt Davy, Malawi, *E. A. Banda et al. 3573* (MO), ITS: KT696045. *M. japira* A. M. G. Azevedo, K. Agostini & M. Sazima, Brazil, *T. M. Moura 630* (UEC), ITS: KT696046, *trn*L-F: KT696101. *M. klitgaardiae* T. M. Moura, G.P. Lewis & A.M.G. Azevedo, Ecuador, *B. Klitgaard 99502* (MO), ITS: KT696047. *M. lamellata* Wilmot-Dear, China, *C. Ford 64* (K), ITS: KT696048, *trn*L-F: KT696102. *M. lamellata* Wilmot-Dear (as *M. paohwashanica* T. Tang & F. T. Wang), China, *X. Q. Wang & Y. N. Xiong 001* (MO), ITS: KT696049, *trn*L-F: KT696103. *M. macrocarpa* Wall., China, *G. Z. Li s.n.* (MO), ITS: KT696050, *trn*L-F: KT696104. *M. macrophylla* Miq., Indonesia, *F. J. A. J. Verheijen 1330/31* (L), ITS: KT696051. *M. macropoda* Baker f., Papua New Guinea, *H. Hopkins s.n.* (L), ITS: KT696052. *M. manongarivensis* Du Puy & Labat, Madagascar, *L. Gautier et al. 3785* (MO), ITS: KT696053, *trn*L-F: KT696105. *M. melanocarpa* Hochst. ex A. Rich., Ethiopia, *I. Friis, W. Abebe & E. Getachew 13442* (K), *trn*L-F: KT696106. *M. mollis* (Kunth) DC., Colombia, *H. Murphy & E. Parra 684* (MO), ITS: KT696054, *trn*L-F: KT696107. *M. mollissima* Teijsm. & Binn. ex Kurz, Papua New Guinea, *J. H. Waterhouse s.n.* (K), ITS: KT696055, *trn*L-F: KT696108. *M. mollissima* Teijsm. & Binn. ex Kurz [as *M. cyanosperma* K. Schum.], Papua New Guinea, *B. Eddie s.n.* (K), ITS: KT696056, *trn*L-F: KT696109. *M. monosperma* (Roxb.) DC., ITS: AB775136.1. *M. mutisiana* (Kunth) DC., Colombia, *J.H. Kirkbride Jr. 2555* (MO), ITS: KT696057, *trn*L-F: KT696110. *M. neocaledonica* Baker f., New Caledonia, *G. McPherson 5261* (K), ITS: KT696058, *trn*L-F: KT696111. *M. nova-guineensis* Scheff., Papua New Guinea, *W.N., Takeuchi* (L 0254121), ITS: KT696059, *trn*L-F: KT696112. *M. poggei* Taub. 01, Malawi, *R-Smith, Pope & Goyder 5836* (K), ITS: KT696060, *trn*L-F: KT696113. *M. poggei* Taub. 02, Zambia, *D. K. Harder et al. 3073* (MO), *trn*L-F: KT696114. *M. pruriens* var. *utilis* (Wall. ex Wight) Baker ex Burck, cultivated in Brazil, *T. M. Moura 994* (UEC), ITS: KT696063, *trn*L-F: KT696118. *M. pruriens* var. *utilis* (Wall. ex Wight) Baker ex Burck (as *M. hassjoo* (Piper & Tracy) Mansf.), Taiwan, *M.T. Kao 10159* (MO), ITS: KT696062, *trn*L-F: KT729516. *M. pruriens* var. *utilis* (Wall. ex Wight) Baker ex Burck 31, Ecuador, *Z. T. Almeida 002* (MO), ITS: KT696064. *M. pruriens* var. *utilis* (Wall. ex Wight) Baker ex Burck 32, Ecuador, *Z. T. Almeida 002* (MO), ITS: KT696065. *M. pruriens* (L.) DC. var. *pruriens*, Venezuela, *R. Ramirez 26* (MO), ITS: KT696061, *trn*L-F: KT729515. *M. pruriens* (L.) DC. 038, French Guiana, *J. J. de Granville* (U 013577), *trn*L-F: KT696115. *M. pruriens* (L.) DC. M7, Mexico, *R. Duno de Stefano1830* (MO), *trn*L-F: KT696116. *M. rostrata* Benth., Brazil, *M. Simon 1639* (CEN), ITS: KT696066, *trn*L-F: KT696120. *M. rostrata* Benth. 36, Peru, *F. Woytkowski 5378* (MO), *trn*L-F: KT729517. *M. samarensis* Merr. 027, Philippines, *C. E. Ridsdale* (L 0396765), ITS: KT696067, *trn*L-F: KT696121. *M. schlechteri* Harms, New Guinea, *R. D. Hoogland 4241* (GH), ITS: KT696068. *M. sempervirens* Hemsl., China, *D.E. Boufford* (L 0254117), ITS: KT696069, *trn*L-F: KT696122. *M. sempervirens* Hemsl., Asia, *Hemsl s.n.* (MO), *trn*L-F: KT696123. *M. sloanei* Fawc. & Rendle, Brazil, *T. M. Moura 1005* (UEC), ITS: KT696070, *trn*L-F: KT696124. *M. stanleyi* C. T. White, Papua New Guinea, *L. J. Brass 24277* (L), ITS: KT729511. *M. stanleyi* C. T. White 2, Papua New Guinea, *Hopkins & Hopkins 1018* (K), ITS: KT696071. *M. stans* Welw. ex Baker, Tanzania, *F. Furuya 95* (MO), ITS: KT696072, *trn*L-F: KT696125. *M. stenoplax* Wilmot-Dear, Thailand, *S. Phusomsaeng & S. Pinnin 49* (K), *trn*L-F: KT696126. *M. stenoplax* Wilmot-Dear, Asia, *T. Kajita s./n.* (FU), ITS: KT696073, *trn*L-F: KT696127. *M. stenoplax* Wilmot-Dear 22, Thailand, *J. F. Maxwell* (L 0401600), *trn*L-F: KT696128. *M. urens* (L.) Medik., Brazil, *T. M. Moura 629* (UEC), ITS: KT696074, *trn*L-F: KT696130. *M. urens* (L.) Medik. 39, Guyana, *T. R. van Andel* (U0085459), *trn*L-F: KT696129. *M. warburgii* K. Schum. & Lauterb., Papua New Guinea, *S. Lenean 1443* (K), ITS: KT696075.

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