NEW DISCOVERIES IN TAXONOMY OF *Dalbergia* GENUS IN GUATEMALA REVEALED BY MOLECULAR APPROACHES †

[NUEVOS DESCUBRIMIENTOS EN LA TAXONOMÍA DEL GÉNERO *Dalbergia* EN GUATEMALA REVELADOS MEDIANTE ENFOQUES MOLECULARES]

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SUMMARY

Background. The *Dalbergia* genus (Fabaceae) in Guatemala harbors valuable rosewood species; however, these timber species face significant threats from illegal logging and deforestation. Due to the morphological similarity between closely related *Dalbergia* species, accurate morphological identification is challenging, leading to uncertainty about the occurrence of new species in the country. The lack of information about the actual number of *Dalbergia* tree species complicates the development of management and conservation strategies for this endangered timber species. **Objective.** To elucidate the taxonomy of the tree species of the *Dalbergia* genus in Guatemala using species molecular delimitation methods. **Methodology**. Sixty-one *Dalbergia* specimens, collected in its natural range in Guatemala, were analyzed using nuclear (ITS) and chloroplast (matK and trnH-psbA) markers. Species delimitation was performed using three methods based on genetic distance, two based on single-locus phylogenetic trees, and two multi-loci. **Results**. Different Molecular Operational Taxonomic Units (MOTU) were estimated, ranging from 2 to 9 depending on the method and locus used. The molecular approaches consistently delimited the 6 species already reported for Guatemala. Furthermore, 3 MOTUs were identified that did not align with these known species, implying the presence of 3 new species for the country. **Implications.** Efficient molecular methods identify *Dalbergia* species from leaf samples, but standardizing wood sample identification is recommended for uncertain wood confiscation origins. This study proposes a new taxonomy of the genus Dalbergia in Guatemala and offers a fast and reliable identification method. **Conclusions.** With the molecular methods used in the study, three new *Dalbergia* species in Guatemala are proposed, corroborating previous suggestions based on morphological characterization. This

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discovery expands the existing inventory of *Dalbergia* tree species in Guatemala, comprising six previously documented species and three novel species that require detailed botanical descriptions for final naming.
Key words: *Dalbergia*; species delimitation; DNA barcoding; molecular markers; conservation.

RESUMEN

Antecedentes. El género *Dalbergia* (Fabaceae) en Guatemala alberga valiosas especies de palo de rosa; sin embargo, estas especies maderables se enfrentan a importantes amenazas por la tala ilegal y deforestación. Debido a la similitud morfológica entre especies de *Dalbergia*, por estar estrechamente relacionadas, la identificación morfológica precisa es un reto, lo que lleva a una incertidumbre actual sobre la ocurrencia de nuevas especies en el país. La información sobre el número real de especies arbóreas de *Dalbergia* es escueta y ello complica el desarrollo de estrategias de gestión y conservación de esta especie maderable amenazada. **Objetivo**. Elucidar la taxonomía de las especies arbóreas del género *Dalbergia* en Guatemala con el uso de métodos de delimitación molecular de especies. **Metodología**. Se analizaron 61 especímenes de *Dalbergia*, colectados en su área de distribución natural en Guatemala, mediante el empleo de marcadores nucleares (ITS) y cloroplásticos (matK y trnH-psbA). La delimitación de especies se realizó utilizando tres métodos basados en distancia genética, dos basados en árboles filogenéticos de un solo locus, y dos multi-loci. **Resultados**. Se identificaron de 2 a 9 Unidades Taxonómicas Operativas Moleculares (MOTU), dependiendo del método y locus empleados. Los enfoques moleculares fueron consistentes para delimitar las 6 especies ya reportadas para Guatemala. Adicionalmente, se encontraron 3 MOTU que no coincidieron con estas especies, lo que sugiere la existencia de 3 especies nuevas para el país. **Implicaciones**. Los métodos moleculares son eficientes para identificar especies de *Dalbergia* a partir de muestras de hojas, pero se recomienda estandarizar la identificación de muestras de madera en casos de orígenes inciertos en incautaciones de madera. Este estudio propone una nueva taxonomía del género *Dalbergia* en Guatemala y ofrece un método de identificación rápido y confiable. **Conclusiones**. Con los métodos moleculares empleados en el estudio, se proponen tres nuevas especies de *Dalbergia* en Guatemala, mismas que fueron sugeridas previamente con caracterización morfológica. Este descubrimiento amplía el inventario existente de especies arbóreas de *Dalbergia* en Guatemala, que comprende seis especies previamente documentadas y tres especies nuevas que requieren descripciones botánicas detalladas para su denominación definitiva.
Palabras clave: *Dalbergia*; delimitación de especies; código de barras de ADN; marcadores moleculares; conservación.

INTRODUCTION

One of the most significant issues in systematics is defining species boundaries, particularly in taxa, where speciation processes have not produced clear morphological differentiation (Goldstein and de Salle 2011). One solution to this issue has been to infer species boundaries by combining other types of information, such as ecology, behavior, biogeography, and genetics (Dayrat, 2005; Fujita *et al*., 2012). When these criteria converge, species boundaries have greater certainty (Fujita *et al*., 2012; Satler *et al*., 2013).

The large-scale biodiversity inventories essential for conservation efforts require quick and accurate species identification (Mace, 2004). Taxonomic identification of tropical trees can be difficult, because closely related species can appear morphologically similar, and individual trees of a species can differ morphologically depending on their age and growing conditions (Parmentier *et al*., 2013). Traditional taxonomic techniques that rely on morphological identifications are expensive and take a long time to produce reliable plant identifications (Gonzalez *et al*., 2009; Costion *et al*., 2011). Additionally, it is very challenging to determine the species when the specimen is broken, fragmented or made of plant materials like leaves, roots, bark, wood, or seeds (Nithaniyal *et al*., 2014).

Dalbergia is a genus that comprises about 250 species of shrubs and trees widely distributed throughout the tropics (Cardoso *et al*., 2013; Vatanparast *et al*., 2013). During the last decades, many revisions based on morphological traits have made taxonomic delimitation of species in *Dalbergia* quite challenging due the similarity between the species (Carvalho, 1997; Sunarno and Ohashi, 1997; Chen *et al*., 2010). Several species in this genus, specially rosewoods, stand out due are widely valued as high-quality timber (Li *et al*., 2017). These rosewoods' heartwood is incredibly hard, dense, and resilient, making it ideal for creating ornaments, musical instruments, carvings, fine furniture and cabinetry (Hassold *et al*., 2016). The high demand for this exceptional wood has led to all *Dalbergia* tree species being protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES); nevertheless, many species are still being targeted

for illegal logging (Espinoza *et al*., 2015; Vardeman and Runk, 2020).

Traditionally, the identification of *Dalbergia* species has been made through morphological (Hartvig *et al*., 2015), chemical and physical characteristics (Wang *et al*., 2016; Wu *et al*., 2017), as well as wood anatomical structures (Gasson, 2011). However, these methods present limitations and are not accurate in species delimitation, mainly in a genus as complex as *Dalbergia* (Sunarno and Ohashi, 1997; Niyomdham, 2002; Chen *et al*., 2010). To overcome these limitations, in recent years, approaches to identify species based on DNA sequences have gained popularity (Carstens *et al*., 2013; Flot, 2015; Cheng *et al*., 2021). DNA barcoding is a method that was initially created to identify species based on sequences from a small standardized DNA region (Hebert *et al*., 2003a; Hebert and Gregory, 2005). DNA barcoding relies on a distance threshold, or barcoding gap, to reveal biodiversity quickly and accurately (Rossini *et al*., 2016).

Methods for species delimitation based on a single locus have increased and are now widely used (Begerow *et al*., 2012; Hebert *et al*., 2003b). Initial studies of DNA barcoding in plants have proposed some coding and non-coding regions in the chloroplast, such as rbcL and trnH-psbA (Kress *et al*., 2005), matK, rpoB, and rpoC1 (Chase *et al*., 2007). Additionally, the recently evolved nuclear area was suggested as potential barcodes, named as nuclear internal transcribed spacer of the ribosomal gene (ITS) (Chen *et al*., 2010). DNA sequences has been successfully used to discriminate between species of genus *Dalbergia* to support conservation strategies (Bhagwat *et al*., 2015; Hartvig *et al*., 2015; Hassold *et al*., 2016; Yu *et al*., 2016), to reveal phylogenetic relationships (Cardoso *et al*., 2013; Vatanparast *et al*., 2013; Li *et al*., 2017; Sotuyo and Pedraza-Ortega, 2022), and describe their biogeography. However, none of these studies include the *Dalbergia* tree species distributed in Guatemala.

In single-locus molecular phylogenies, the Poisson Tree Processes (PTP) approach is designed for species determination. It employs rooted, bifurcated, albeit not always ultrametric trees. It is predicated on the idea that branch lengths vary depending on whether or not speciation dynamics or merely mutation and drift impact them, and that the two classes of branches are distinguished by switch points on a tree, the locations of which are determined using maximum probability (Zhang *et al*., 2013). The PTP model is the base for the Bayesian Poisson Tree Processes (bTPT) and multiple rate PTP (mPTP). The Bayesian support (BS) values are added to the delimited species on the input tree in the bPTP algorithm, which is an updated version of the original maximum likelihood PTP.

Recent developments in mPTP enable lineage-specific distributions of intra-specific branch lengths. The mPTP non-coalescent technique simulates the branching processes with the presumption that branching events within a species will be more frequent than those across species. The variation in intra-specific branch lengths is important in the mPTP model, because it allows for a more realistic representation of the evolutionary processes within and between species. By considering this variation, the mPTP model can more accurately distinguish between intra-specific and inter-specific processes, as it assumes that the rate of coalescence within a species is higher than the rate of speciation. Incorporating the variation in intra-specific branch lengths helps the model identify the transition points between these processes, leading to a more precise delimitation of species boundaries, and avoiding oversimplification of the evolutionary processes. This approach takes into account the possibility for intraspecific diversity to diverge (Kapli *et al*., 2017). Intraspecific diversity refers to the genetic variation that exists within a single species. The mPTP model accounts for intraspecific diversity by allowing for variation in the rate of coalescence (lineages merging into a common ancestor) among different lineages or species. Intraspecific diversity is reflected in the branch lengths within a species on the phylogenetic tree, with shorter branches indicating higher coalescence rates and longer branches indicating higher intraspecific diversity.

In order to determine if two individuals belong to same species, the Automatic Barcode Gap Discovery (ABGD) seeks to find a distinctive barcode gap, or a threshold of genetic distance (Puillandre *et al*., 2012). The threshold value that distinguishes between intra- and inter-specific divergence levels is determined by a pairwise distance matrix in the model. The sequences in the dataset are compared in pairs, and if their nucleotide divergence is less than this cutoff, they are included in the same Molecular Operational Taxonomic Unit (MOTU). All sequences having a nucleotide divergence below the threshold are combined to form sets of sequences.

The Assemble Species by Automatic Partitioning (ASAP) is based on an approach that solely computes paired genetic distances, which speeds

up phylogenetic reconstruction (Puillandre *et al*., 2021). To do this, the method ranks dissimilarity matrix's values in ascending order, with each value acting as border value between degrees of intra- and inter-specific divergence. After that, a score is given to a variety of alternative sequence groupings. This rating is determined by two factors: the likelihood that a set of sequences represents a species (p-value), and the distance, in bars, between group's current state and its state prior to grouping (W). The p-values and W-values are then ordered in ascending and descending fashion, respectively.

In Guatemala there is still no accurate inventory of the tree species of *Dalbergia* genus; for this reason, in recent years, field expeditions have been carried out in the country to record all *Dalbergia* tree species. All collected specimens have been exhaustively examined through morphological characters, with the use of specialized taxonomic key for *Dalbergia* species distributed in Mexico and Central America (Linares and Sousa, 2007). Nevertheless, some specimens do not conform to any species, so morphological evidence suggests the presence of new *Dalbergia* tree species in Guatemala; nonetheless, there is still botanical confusion in their identification (Linares *et al*., 2022).

Thus, the objective of this study was to elucidate the taxonomy of tree species of the *Dalbergia* genus in Guatemala using widely reported species molecular delimitation methods. To achieve the objective sequences of three DNA markers (ITS, matK, and trnH-psbA) were used in specimens of *Dalbergia* genus collected in its natural distribution zones in Guatemala. This study hypothesizes that the application of molecular methods will delineate six species previously documented in Guatemala. It will also define three newly discovered species in the country using chloroplast and nuclear molecular markers. It will represent a step forward to improve the conservation effort of *Dalbergia* tree species.

MATERIALS AND METHODS

Sampling

During 2021 and 2022 field expeditions were conducted in the distribution areas of tree species of *Dalbergia* genus in Guatemala. In these, 61 specimens were collected (Figure 1) and based on specialized taxonomic keys for *Dalbergia* sp. distributed in Mexico and Central America (Linares and Sousa, 2007), *Dalbergia calderonii* (5 specimens), *D. calycina* (5), *D. melanocardium* (1), *D. retusa* var. *cuscatlanica* (7), *D. stevensonii* (7), and *D. tucurensis* (18) were identified. In addition, three species of *Dalbergia* were morphologically identified as putative new species, being named as *D.* aff. *tucurensis* (12), *D.* aff. *calycina* (4), and *D.* aff. *retusa var cuscatlanica* (2); these species were differentiated morphologically from all other described by the taxonomic keys. For molecular analyses, five healthy and young leaves were collected from each species and stored in plastic bags with silica gel for dehydration.

DNA extraction, amplification and sequencing

Total genomic DNA were extracted from 20 mg of silica gel dried leaves using the Cetyltrimethylammonium Bromide (CTAB) method (Doyle and Doyle, 1987). ITS, matK, rbcL and trnH-psbA loci PCR amplifications were done following the methods described by Bhagwat *et al*. (2015). Amplifications were carried out on a Bio Rad® PTC-200 Thermal Cycler. PCR products were purified and sequenced by Psomagen Inc. (Maryland, USA).

Sequence analysis

Bidirectional sequences were assembled, trimmed primer sequences on both ends, and manually corrected to generate consensus sequences using Geneious Prime v.2022.1.2 program (Kearse *et al*., 2012). Consensus sequences were generated for all molecular markers (ITS, matK, and trnH-psbA), to improve the accuracy and reliability of the sequencing data for downstream analyses by resolving discrepancies between forward and reverse sequencing reads. The edited sequences were aligned using MAFFT v.7.4 method (Katoh and Standley, 2013) implemented in UGENE v.1.32.0 program (Okonechnikov *et al.*, 2012). Subsequently, the multiple alignment was curated using the BMGE program (Criscuolo and Gribaldo, 2010), to remove ambiguously aligned regions and highly variable (saturated) characters, ensuring the use of homologous and reliable sequences for phylogenetic reconstruction and species delimitation methods. This step was necessary because ITS and trnH-psbA markers exhibited larger incongruences due to their higher variability or the presence of insertions/deletions. The removal of these problematic regions improves the performance of phylogenetic reconstruction by reducing noise and potential homoplasy in the data, resulting in more reliable and informative characters for accurate and well-resolved phylogenies.

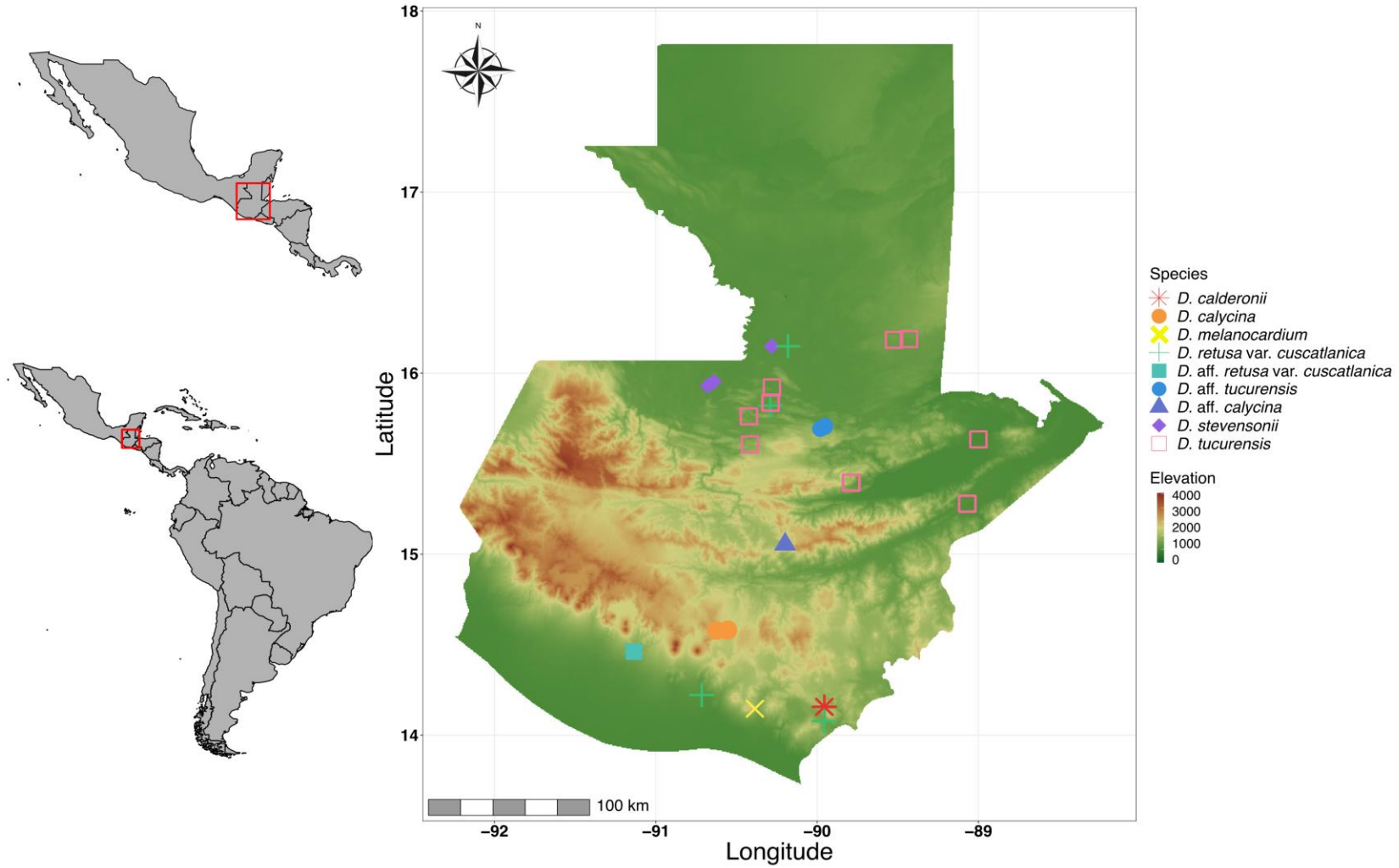

Figure 1. Geographical location of *Dalbergia* species sampled in Guatemala. Source: own elaboration.

Phylogenetic analysis

Since the molecular delimitation methods of multi-rate Poisson Tree Processes (mPTP), Bayesian Poisson Tree Processes (bPTP) and Bayesian General Mixed Yule-Coalescent Model (bGMYC) require a phylogenetic tree as input file, phylogenetic reconstructions were first developed. The selection of nucleotide substitution model was done based on Bayesian informative criterion (BIC) using ModelFinder (Kalyaanamoorthy *et al*., 2017). The nucleotide substitution models selected for ITS, matK and trnH-psbA were TN+G, TPM3 and F81, respectively. These evolutionary models were used to obtain three phylogenetic trees for each marker as follow: Maximum Likelihood (ML) in IQ-Tree (Nguyen *et al*., 2015), Bayesian Inference (BI) in Mr. Bayes v.3.2.6 (Ronquist and Huelsenbeck, 2003), and ultrametric gene tree in BEAST2 v.2.6.3 (Bouckaert *et al.*, 2014). The three software programs were included into CIPRES Science Gateway interface (Miller *et al*., 2012). The phylogentic trees were used as input files for species delimitation methods (see below).

Branch support was computed in ML analysis using 10000 Ultrafast Bootstrap repetitions (UFBoot; Minh *et al*., 2013) and GENESITE resampling technique, which permits resampling of partitions and sites within partitions (Gadagkar *et al*., 2005).

Eight chains in two independent runs with10 million Metropolis Coupled Markov Chain Monte Carlo (MCMC) generations were employed for BI analysis. The model's default settings were not changed, and the trees were sampled every 1000 generations after a random tree was used to start the analysis. The probability parameters in Tracer v1.7.1 software (Rambaut *et al.*, 2018) were used to confirm the convergence and seasonality of each run, after which all trees before stationarity phase were discarded as burn-in. By creating a majority-rule consensus and using the trees that were not rejected as burn-in, the posterior probability (pP) was derived for each individual node. To create an ultrametric gene tree the calibrated birth-death model with an uncorrelated lognormal relaxed clock (ULRC) were applied, and all other priors left at default values. The analyses were performed using two runs of 10 million generations each, and trees sampled every 1,000 generations. Convergence of independent runs and effective sample size (appropriate ESS > 200) were assessed using Tracer v1.7.1 (Rambaut *et al*., 2018). All trees prior stationary phase were discarded as burn-in, and a maximum clade credibility tree was created in TreeAnotator v2.6.2 (Bouckaert *et al*., 2014) using mean heights for annotation.

Molecular species delimitation

For the creation of species hypotheses in this work, three distance-based and two tree-based single locus, and two multi-loci species delimitation methods were applied, as follows: 1) bayesian Poisson Tree Processes (bPTP; Zhang *et al*., 2013); 2) multi-rate PTP (mPTP; Kapli *et al*., 2017); 3) bayesian General Mixed Yule-Coalescent Model (bGMYC; Reid and Carstens, 2012); 4) Automatic Barcode Gap Discovery (ABDG; Puillandre *et al*., 2012); 5) Assemble Species by Automatic Partitioning (ASAP; Puillandre *et al*., 2021); 6) Species Tree and Classification Estimation Yarely (STACEY; Jones, 2017) and 7) haplotype network. Putative new species were delineated using a variety of methods, which improves the interpretation of the findings, since method congruence encourages the identification of new taxonomic entities. According to earlier studies, employing different approaches simultaneously to define a species boundary enables the strategies to balance out each other's shortcomings (Kekkonen and Hebert, 2014; Mutanen *et al*., 2015; Ji *et al.*, 2021)

The bPTP and mPTP methods used as input the ML and BI trees topologies and the model specifications and MCMC configurations set by default in the bPTP web interface (bPTP server, 2013) and the Exelisis Lab platform (The Exelixis Lab, 2013), respectively.

The bGMYC coalescent technique assumes that, at the branch points of a tree, one of two events—coalescence events between lineages within a species or divergence events between two species (speciation)—occurs (Zhang *et al*., 2013). Using LogCombinerv2.6.3 from BEAST2 package, the last 100 ultrametric trees obtained from calibrated birth-death model with an uncorrelated lognormal relaxed clock were chosen and utilized as input data. The delimitation analysis was developed in the bGMYC package (Reid and Carstens, 2012), implemented in R v.4.2.0 (R Core Team 2022) with the input parameter as follows: mcmc = 100,000, burnin = 9,000, thinning = 100, t1 = 6, t2 = 12 (considering outgroup and top range of predicted mPTP species), py1 = 0.5, py2 = 1.5, pc1 = 0.1, pc2 = 0.5, start = c (1.0, 0.1, 11), scale = c (20, 10, 5.00).

The Kimura two-parameter substitution model, 50 recursive steps, a gap width (X) of 1, and maximum value of intraspecific divergence between 0.001 and 0.1 were used as default to analyze the alignments in ABGD web (ABGD web, 2023). The gap width parameter in ABGD determines the interval between values of prior intraspecific divergence that the method explores to find the barcode gap. A gap width of 1 is commonly used because it allows for a fine-grained, step-by-step exploration of the prior intraspecific divergence values from 0.001 to 0.1 in increments of 0.001. This increases the likelihood of detecting the optimal threshold separating intraspecific from interspecific divergences. While the optimal gap width can vary by dataset, a value of 1 is a reasonable default choice, balancing computational efficiency with sufficient precision in estimating the barcode gap. ASAP web interface was used (ASAP web, 2023) to analyze the alignments.

Combined DNA sequence data (ITS + matK + trnH-psbA) and STACEY coalescent approach were utilized to identify new potential species. Both a guide tree and a priori species assignment are not necessary in this multiple-loci technique. Furthermore, the minimum cluster tree's delimited species number (which ranges from one to whole number of terminals) can be variable (Jones, 2017). Being implemented as a package within BEAST2, the STACEY analysis makes use of birth-death-collapsed tree model as a prior. To summarize the tree posterior distribution and determine the frequency with which each pair of taxa were allocated to the same clade, the SpeciesDelimitationAnalyzer software was used for this analysis. As input data, STACEY's species.tree file was utilized with similarity cutoff set to 1.0 and burning set to 1000.

DnaSP 6 v6.12 (Rozas *et al*., 2017) was used to identify unique haplotypes on concatenated matrix of 2 chloroplast loci and ITS loci, and for exploring relationships amongst them an haplotype network was constructed with the TCS network method (Templeton *et al*., 1992) using PopART v1.7 (Leigh and Bryant, 2015). The robustness of haplotype clusters was evaluated by ML analysis using 10000 Ultrafast Bootstrap repetitions under IQ-Tree, and under BI using Mr. Bayes v.3.2.6 with same parameters detailed in the Phylogenetic analysis section (see above). For both analysis a GTR+G model was used.

Assessment of DNA barcodes for species identification

To evaluate the accuracy of each marker on species identification the best match approach (BM) *(*Meier *et al*., 2006) was employed. In this method, each sequence from the dataset was used as a query against the remaining sequences from same dataset. With BM, a query sequence was identified for searching reference sequence and identify the best match with smallest genetic distance to query (Meier *et al*., 2006). If subject and query sequences belonged to same species, the identification was deemed successful. Alternatively, the samples were deemed ambiguous, if more than one query sequence from several species showed an equally excellent match. Previous to employing BM analysis, the sequences were named according to best partition, based on accordance among delimitation methods and morphological identification. Ad hoc package (Sonet *et al*., 2013) implemented in R was used to conduct BM analysis. The genetic distance was calculated based on the nucleotide substitution models previous identified for each marker and combined data set, between species based on partition already described using MEGA X (Kumar *et al*., 2018).

RESULTS

Properties of molecular dataset

The nuclear DNA region ITS, the chloroplast DNA region matK, and the chloroplast trnH-psbA intergenic spacer were successfully amplified and exhibited different values of variable sites and genetic distances (Table 1).

The number of sequences obtained from each marker varied depending on the success of PCR amplification. Forty-nine DNA samples successfully amplified for all three markers (Table 1). The phylogenies obtained with each marker and the number of entries according to amplification success are shown in appendix section. Despite having different numbers of entries, the tree topologies were concordant for all markers.

Table 1. Evaluation of three DNA markers for *Dalbergia* species identification.

	ITS	matK	trnH-psbA	ITS + matK + trnH-psbA
PCR success rate (%)	95	88.5	100	-
Sequencing success rate (%)	100	100	100	-
Number of sequences analyzed	58	54	61	49
Aligned sequence length (bp)	624	836	296	1756
Variable sites	106 (16.98 %)	14 (1.67 %)	16 (5.41 %)	136 (7.75 %)
Parsimony-informative sites	99 (15.87 %)	11 (1.32 %)	16 (5.41 %)	126 (7.18 %)
Mean intraspecific distance (range)	0.21 (0 – 1.34)	0.02 (0 – 0.24)	0.33 (0 – 2.76)	0.16 (0 – 1.21)
Mean interspecific distance (range)	9.14 (1.95 – 13.7)	0.52 (0 – 0.11)	3.01 (0 – 5.01)	5.09 (0.29 – 10.84)

Intra and interspecific values are expressed in percentage.

Phylogenetic relationships

BI and ML analyses yielded identical topologies for all DNA markers. The phylogeny recovered from ITS data exhibited two main clades well supported (Figure S1). The first included *D. retusa* var *cuscatlanica*, *D. calycina*, *D.* aff. *calycina* and *D.* aff. *retusa var cuscatlanica*. The second clade comprised *D. tucurensis*, *D. melanocardium*, *D. calderonii*, *D. stevensonii* and *D.* aff. *tucurensis*. The obtained topology for matK (Figure S2) and trnH-psbA loci (Figure S3) was consistent to ITS; nonetheless, the phylogeny from matK exhibited polytomies and the relationships among the second clade species' was not completely resolved. Finally, the complete data set (ITS + matK + trnH-psbA) (Figure S4) supported the presences of two main clades with same species distribution already described. The phylogeny among species was highly supported (pP > 0.9, BS > 75).

Performance of molecular species delimitation methods

Results from species-delimitation methods were found to be congruent (Figure 2). Benchmarked against morphologically differentiable taxa, the majority of methods supported the hypothesis of six species, previously documented in Guatemala and also define three newly discovered species.

Concerning ITS data, all methods based on single loci bPTP, mPTP, bGMYC, ABGD and ASAP suggested six OTUs. The results for ITS were not consistent with morphological identification, due the delimitation methods included *D.* aff. *tucurensis* and *D. tucurensis* as same species. Similar situation results with *D.* aff. *calycina* wich was identified as same species with *D. calycina*, while *D.* aff. *retusa var cuscatlanica* was not separated from *D. retusa* var *cuscatlanica.* It is important to highlighted that all delimitation methods identified to *D. melanocardium* as a unique species.

Regarding matK data set, the species delimitation methods found two partitions. The first one included eigth OTUs. For this partition, bPTP, mPTP and ASAP methods were congruent each other, and suggesting the separation of *D.* aff. *tucurensis* from *D. tucurensis*, *D.* aff. *calycina* from *D. calycina, and D.* aff. *retusa var cuscatlanica* from *D. retusa* var. *cuscatlanica*. The second partition obtained by ABGD method exhibited only two OTUs, corresponding with main clades displayed through the phylogeny (Figure 2). Both partitions included *D. melanocardium* in same as *D.* aff. *tucurensis.*

Respecting trnH-psbA loci, three clustering models were found. The first partition was exhibited by bGMYC method and included seven OTUs corresponding to *D. calderonii*, *D. tucurensis*, *D. stevensonii*, *D. calycina*, *D.* aff. *tucurensis*, *D.* aff. *calycina*. The last group comprised *D. retusa* var. *cuscatlanica* and *D.* aff. *retusa var cuscatlanica* as the same species. The second partitions was proposed by bPTP and mPTP methods. The only difference with the first partition was the splitting of *D. retusa* var *custlanica* and *D.* aff. *retusa var cuscatlanica* as different species. Finally, with the last partition, suggested by ABDG and ASAP, six OTUs were found. For this case, this partition enclosed *D.* aff. *calycina*, *D.* aff. *retusa var cuscatlanica* and *D. retusa* var *cuscatlanica* as one species. The remaining OTUs were adjusted to bPTP and mPTP partition. All delimitation methods embodied *D. melanocardium* in the same OTU as *D.* aff. *tucurensis.*

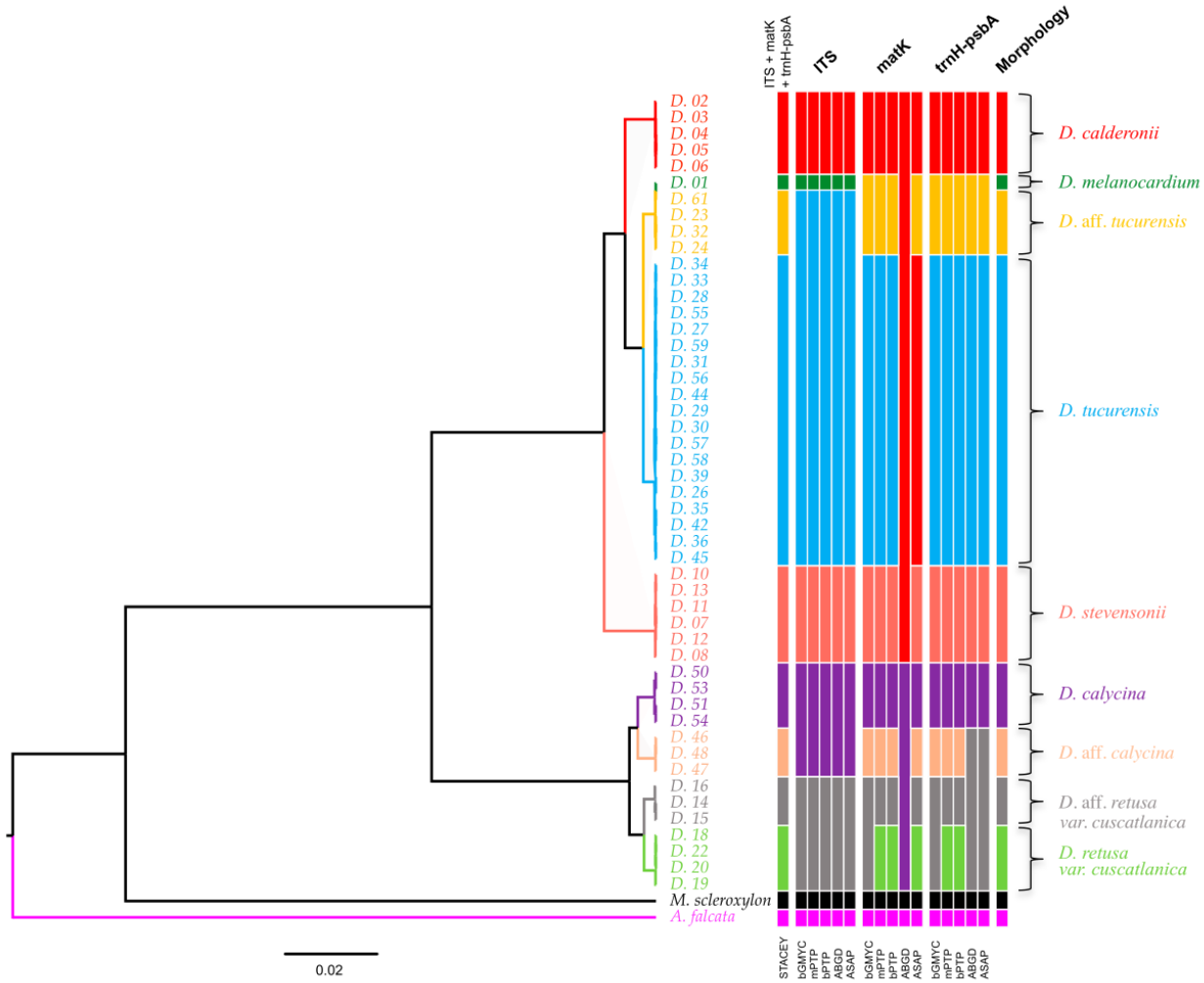

Figure 2. Schematic representation of the results of species delimitation methods in the genus *Dalbergia* (49 individuals). The results of the multilocus method STACEY are compared with the results of the single-locus methods bGMYC, mPTP, bPTP, ABGD, ASAP. The results of the different methods are represented with colored bars highlighting the congruence between methods. The ultrametric tree presented is derived from the STACEY analysis (concatenated loci) and is solely utilized for illustrating the outcomes of the different methods.

The multi-loci species delimitation method STACEY proposed nine OTUs corresponding to *D. calderonii*, *D. melanocardium*, *D. tucurensis*, *D. stevensonii*, *D. calycina*, *D. retusa* var *cuscatlanica*, *D.* aff. *tucurensis*, *D.* aff. *calycina*, and *D.* aff. *retusa var cuscatlanica*. This partition was congruent with morphological identification. Both phylogenetic trees, ITS nucleotypes and cpDNA haplotypes, indicated that nucleotypes/haplotypes can be separated into nine main groups with strong bayesian probabilities and bootstrap (> 0.98, > 95) (Figures 3a,4a). The nucleotype/haplotype network of nuclear DNA and cpDNA was concordant with phylogenetic relationship supporting a division into nine divergent haplogroups (Figures 3b, 4b). With exception of *D. tucurensis*, *D. calycina*, and *D.* aff. *retusa var cuscatlanica*, all species were represented by an unique haplotypes.

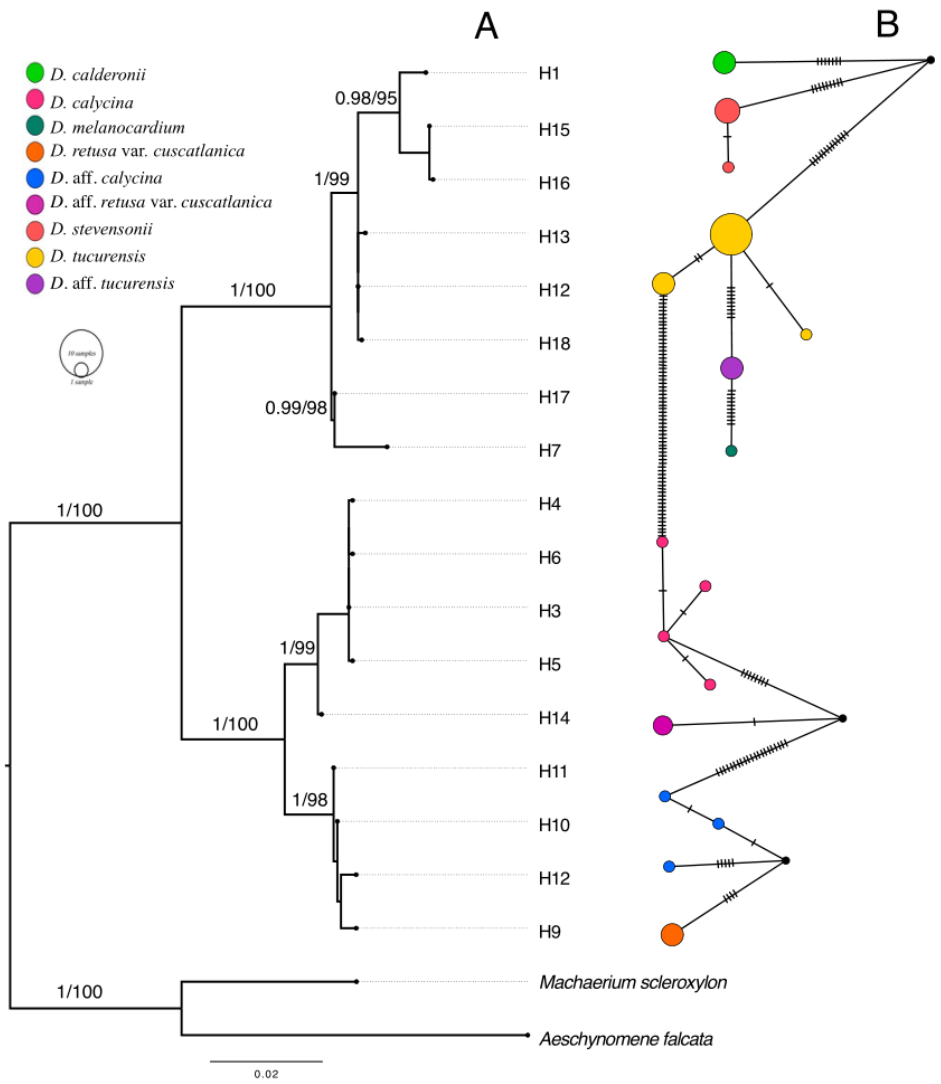

Figure 3. Phylogenetic relationships and haplotype network based on matK and psbA-trnH haplotypes of *Dalbergia* tree species. (a) Phylogenetic tree from Bayesian inference analysis with Bayesian posterior probabilities/maximum likelihood bootstrap values above branches; and (b) Haplotype network based on the TCS method. Hash marks indicate the number of mutations, size of the circle represents the number of observed haplotypes and black circles indicate unsampled or extinct haplotypes.

Specimens identification through BM

The performance for specimens identification by DNA barcoding was tested by BM method indicated above. The rate of correct ambiguous and incorrect identification for each locus and combined data are presented in Figure 5. The best identification success was obtained when using combined data (ITS + matK + trnH-psbA) with 98.03% of correct identification, whereas, ITS loci exhibited the lowest correct identification (49.13 %). For each locus, the list of species responsible for ambiguous identifications were generated (see S1 Table for details).

DISCUSSION

An important challenge in biological research is taxonomic identification of species, since a species' unclear taxonomy makes it difficult to study its biology. The "taxonomic obstacle" is defined as restrictions on the description and identification of

new species (Sabadini *et al.*, 2020). The tree species of genus *Dalbergia* is an example of a taxon with this difficulty, because all species of *Dalbergia* in Guatemala are considered to be endangered or at risk of extinction, and an accurate delimitation is crucial for effective conservation and management of these species. Accurate species identification can help to identify areas of high conservation priority and guide conservation efforts.

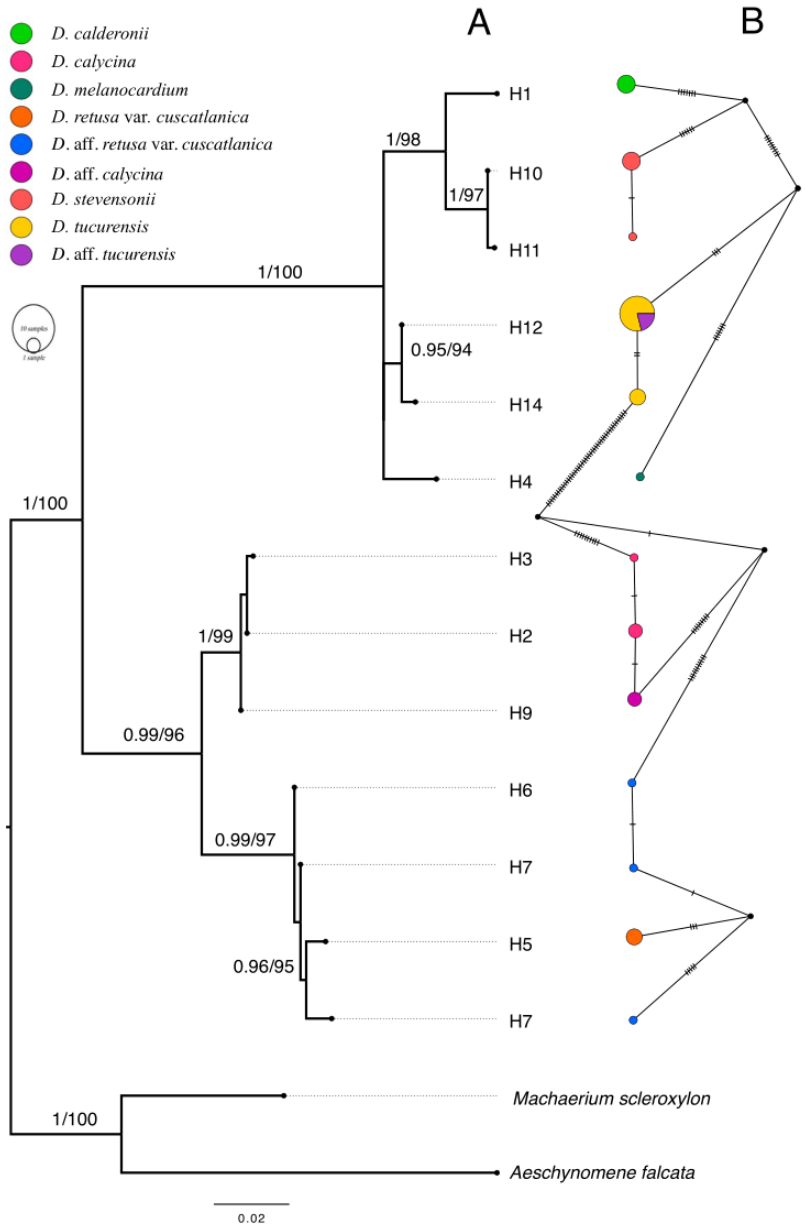

Figure 4. Phylogenetic relationships and haplotype network based on ITS haplotypes of *Dalbergia* tree species. (a) Phylogenetic tree from Bayesian inference analysis with Bayesian posterior probabilities/maximum likelihood bootstrap values above the branches; and (b) Haplotype network based on TCS method. Hash marks indicate the number of mutations, size of the circle represents the number of observed haplotypes and black circles indicate unsampled or extinct haplotypes.

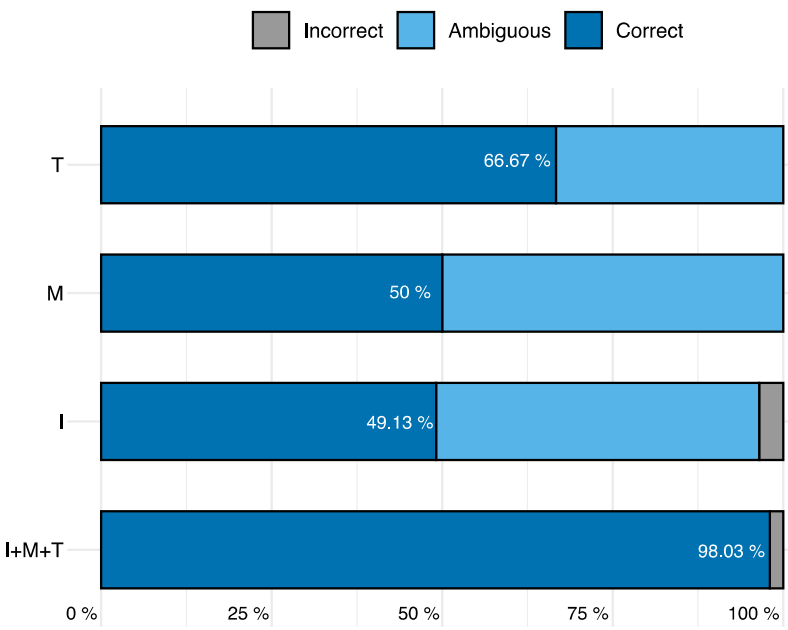

Figure 5. Species identification achievement of DNA barcode in *Dalbergia* tree species based on analysis of "best match" for the three DNA barcodes and combined data. The letters on y-axis represent different barcoding: I: ITS; M: matK; T: trnH-psbA.

Morphologically, the existence of three new species in Guatemala was suspected (Linares *et al*., 2022), which was confirmed by molecular data obtained. The results of this study suggest that a combination of ITS, matK and trnH-psbA loci can effectively identify *Dalbergia* species. The high success rates for PCR amplification (95% for ITS, 88.5% for matK, and 100% for trnH-psbA), and high levels of variable and parsimony-informative sites in final alignments indicate that, these loci contain enough genetic variation to distinguish between Guatemalan *Dalbergia* tree species. The mean intra and interspecific distances, calculated in this study are consistent with previous studies that have used similar loci and methods to delimit *Dalbergia* species. For example, Hassold *et al*. (2016) found that the mean interspecific distances between *Dalbergia* species at ITS and matK regions ranged from 11.1 to 4.61%, respectively. Yu *et al*., (2017) found that interspecific distances between *Dalbergia* species at ITS and trnH-psbA regions ranged from 7.2 to 1.7 %. The intraspecific distances are also consistent with these studies and found that the intraspecific distances were lower than interspecific distances, which indicates that these loci are capable of distinguishing between *Dalbergia* species. The intraspecific genetic distance is also a useful tool for species delimitation, as it can be used to identify the threshold value that separates individuals within same species, which is based by ABGD method (Puillandre *et al*., 2012).

The results showed that, the use of ITS sequences in combination with matK and trnH-psbA loci provides a robust and well-supported phylogenetic tree for *Dalbergia* species analyzed in this study. This is a common practice in phylogenetic analysis as it allows for the incorporation of multiple sources of information to infer evolutionary relationships. The use of multiple DNA markers can increase the amount of variation used in the analysis and can help to overcome limitations of any single marker, resulting in well supported phylogenetic reconstructions on *Dalbergia* genus likewise (Rahaingoson *et al*., 2022). In addition, another explanation for the success of this marker combination is that ITS, matK, and trnH-psbA loci are located in different parts of the genome, and may have different levels of variation and evolutionary patterns (Bieniek *et al*., 2015).

The polytomies or unresolved branches in the tree generated by matK marker means that the relationships among certain species were not completely resolved. This is not an uncommon

occurrence when using a single DNA marker for phylogenetic analysis. One possible explanation for the polytomies observed in matK locus is that, it may not have sufficient variation to fully resolve the relationships among all species in *Dalbergia* genus. Aditionally, matK gene have been widely reported for resolving taxonomic issues at the family and genus levels in plant taxonomy (Hilu and Liang, 1997; Cuénoud *et al*., 2002; Hilu *et al*., 2003; Müller *et al*., 2006). Similar situation was with other genus as *Dioscorea* (Sun *et al*., 2012) and *Myristica* (Tallei and Kolondam, 2015). Another possible explanation is that the matK locus may not be evolving at the same rate across all species in *Dalbergia* genus. This could lead to some species diverging more rapidly than others, making it difficult to fully resolve their relationships using this marker alone (Gagnon *et al*., 2022).

Among the species delimitation methods used in this study, the multi-rate Poisson Tree Processes (mPTP) and the Bayesian Poisson Tree Processes (bPTP) consider differences in branch lengths. These methods assume that the number of substitutions between species is significantly higher than the number of substitutions within species. They use the branch lengths of the phylogenetic input tree to estimate the transition points between inter- and intraspecific processes, thus delimiting species boundaries (Zhang et al., 2013; Kapli et al., 2017).

In the case of the matK marker, if there is insufficient variation or if the locus is evolving at different rates across species, it may lead to short branch lengths and unresolved relationships (polytomies) in some parts of the tree. This can make it challenging for mPTP and bPTP methods to accurately delimit species boundaries, as they rely on the assumption that branch lengths reflect the transition between speciation and coalescent processes. The lack of resolution in the matK tree may lead to an underestimation or overestimation of the number of species by these methods.

It is worth noting that these results are consistent with other phylogenetic studies of *Dalbergia* genus that have used a combination of DNA markers, including nuclear and chloroplast loci. For example, Sotuyo and Pedraza-Ortega (2022) and Li *et al*. (2017) used a similar combination of markers, and found a similar phylogenetic structure for *Dalbergia* genus. However, it is important to note that, the results of different studies may vary depending on specific species included and methods used.

Molecular species delimitation methods were used to examine the phylogenetic reconstructions and support the delimitation of *D.* aff. *tucurensis*, *D.* aff. *calycina*, and *D.* aff. *retusa* var. *cuscatlanica*. These methods are designed to infer species boundaries, which is particularly useful for distinguishing closely related species that may have similar morphological or behavioral characteristics (Dumas *et al*., 2015; Guo and Kong, 2022). In contrast, phylogenetic analysis is primarily used to infer evolutionary relationships but not specifically designed to infer species boundaries (Young and Gillung, 2020).

The approaches used herein estimated several MOTUs ranging from 2 to 9, depending on the method and locus employed. The molecular approaches were able to delimit most of the Dalbergia species, although the ITS, matK, and trnH-psbA datasets showed some inconsistencies with morphological identification. This variability in clustering patterns underscores the complexity of species differentiation and emphasizes the value of a multi-locus approach in molecular species delimitation (Vitecek, 2017).

The STACEY method proposed nine MOTUs corresponding to different Dalbergia species, consistent with morphological identification and supporting the hypothesis of three new tree species in the Dalbergia genus from Guatemala. Multi-loci methods provide a more robust and reliable estimation of species boundaries by incorporating information from multiple genetic markers (Zimmerman *et al*., 2020), accounting for effects of genetic drift, gene flow, and hybridization (Tsykun *et al*., 2017), and resolving complex cases of reticulation (Petzold and Hassanin, 2020).

The application of network haplotypes as a species delimitation method successfully identified and distinguished closely related species within genus Dalbergia, as evidenced by the separation of nucleotypes/cp DNA haplotypes into nine main groups with high levels of genetic divergence and concordance with morphological identification (Liu *et al*., 2015; Wei *et al*., 2017).

DNA barcoding using combined data from ITS, matK, and trnH-psbA markers resulted in the highest identification success, while single locus analyses showed a significantly lower correct identification rate. These results highlight the

importance of using multiple markers in DNA barcoding to increase the accuracy of species identification, particularly for endangered species (Yu *et al*., 2016; He *et al*., 2019).

CONCLUSION

The molecular species delimitation methods support the hypothesis of three new putative *Dalbergia* species in Guatemala, tentatively named like *D.* aff. *tucurensis*, *D.* aff. *calycina*, and *D.* aff. *retusa* var *cuscatlanica*, and these were suggested by previous morphological characterization. This discovery expands the existing inventory of *Dalbergia* tree species in Guatemala, comprising six previously documented species and three novel species that require detailed botanical descriptions for final naming.

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Conflict of interests. The authors declare that there is no conflict of interest related to this publication.

Compliance with ethical standards. No ethical approval was required as any animal or feed was not used in this study.

Data availability. Data are available with José Alejandro Ruiz-Chután (email:josealejandro.ruiz@icloud.com), upon reasonable request

Author contribution statement (CRedit). J.A. Ruiz-Chután – Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing., **M. Herrera –** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – original draft., **M. Kalousová –** Writing – original draft., Writing – review & editing., **J. Linares –** Investigation, Methodology., **B. Lojka –** Writing – original draft., Writing – review & editing., **S. Barrios –** Writing – original draft., Writing – review & editing., **P.E. Choxom-Chamorro –** Investigation, Methodology., **L.E. Velásquez-Méndez –** Investigation, Methodology., **A. Sánchez-Pérez –** Investigation, Methodology., **J.E. Berdúo-Sandoval –** Data curation, Investigation, Methodology, Writing – original draft.

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SUPPLEMENTARY MATERIAL

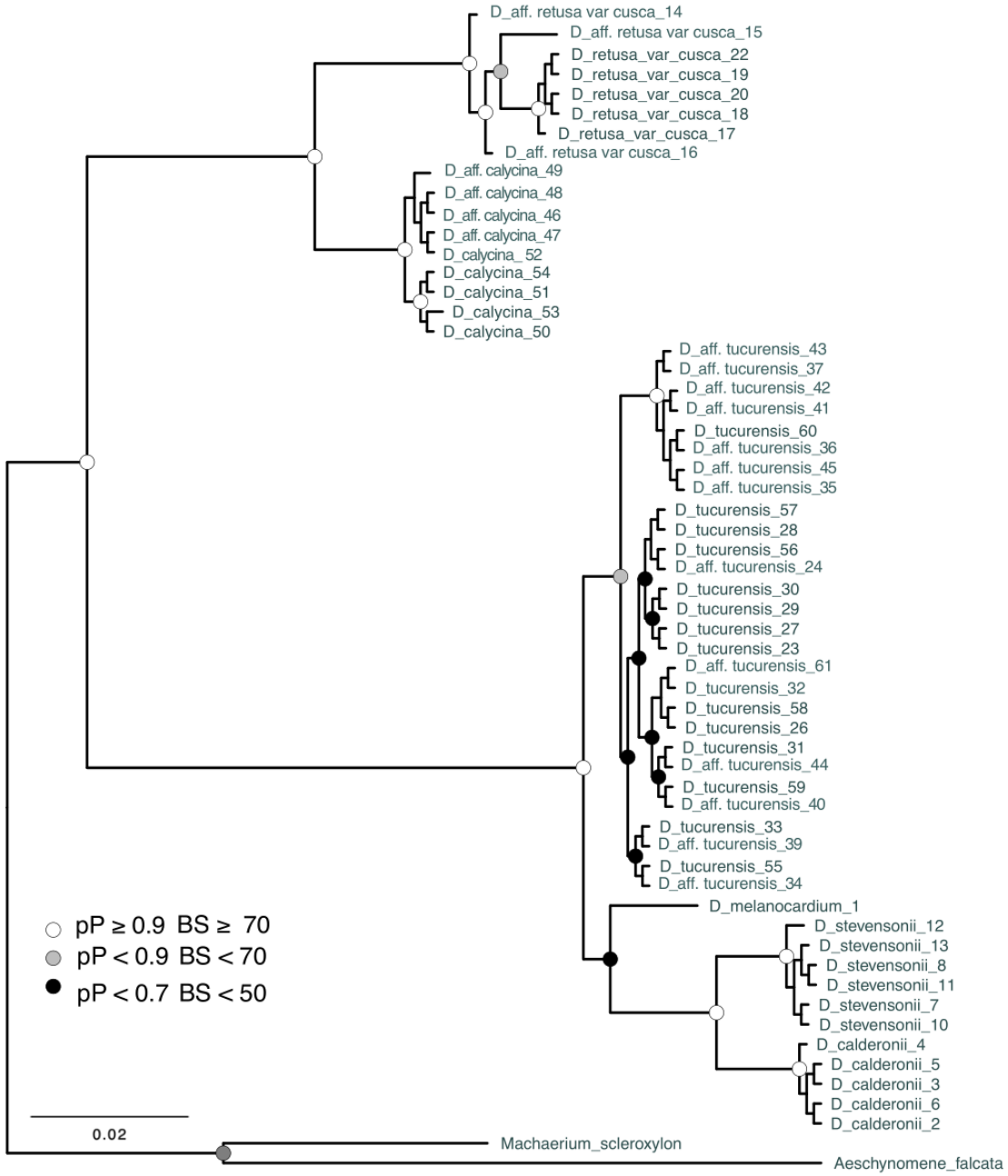

Figure S1. Phylogenetic relationships among tree species of the *Dalbergia* genus based on sequences data of ITS marker, Nodal support for BI/ML analysis is represented by the color of the circles. Nodes without circles reported BI/ML values lower than 0.7/50. Tip labels according to morphological identification. pP: posterior probability, BS: bootstrap support.

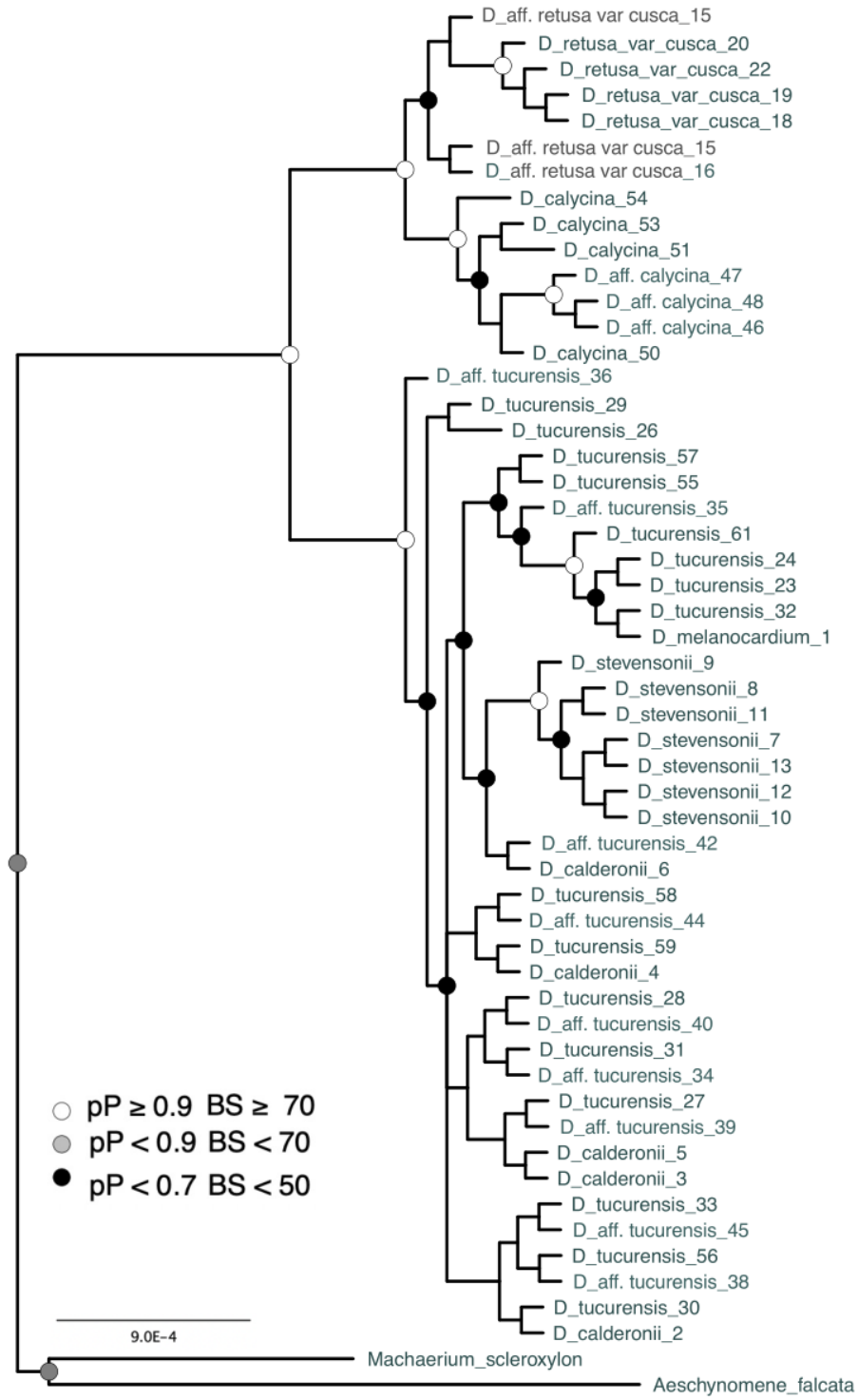

Figure S2. Phylogenetic relationships among tree species of the *Dalbergia* genus based on sequences data of matK marker. Nodal support for BI/ML analysis is represented by the color of the circles. Nodes without circles reported BI/ML values lower than 0.7/50. Tip labels according to morphological identification. pP: posterior probability, BS: bootstrap support.

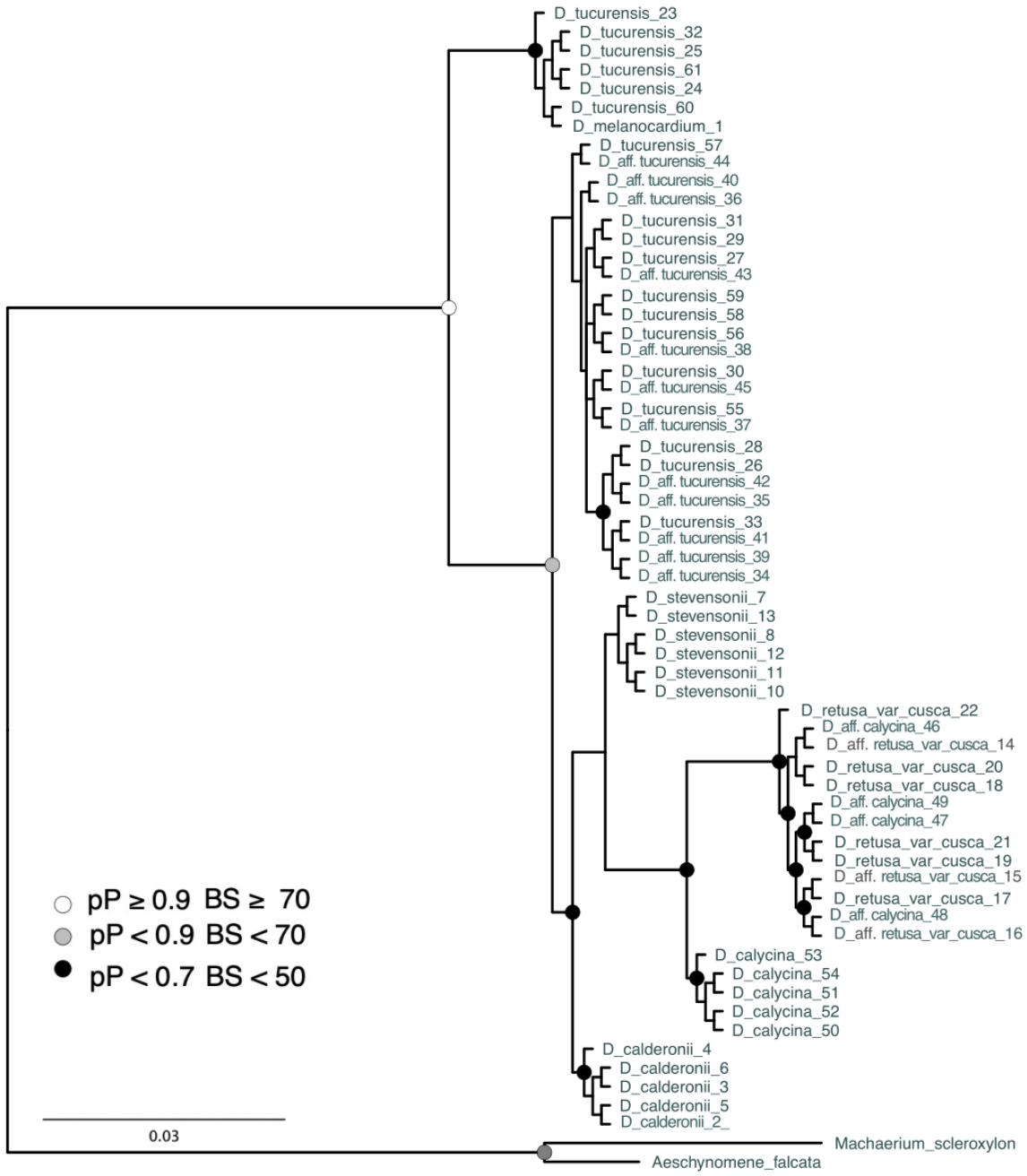

Figure S3. Phylogenetic relationships among tree species of the *Dalbergia* genus based on sequences data of trnH-psbA marker. Nodal support for BI/ML analysis is represented by the color of the circles. Nodes without circles reported BI/ML values lower than 0.7/50. Tip labels according to morphological identification. pP: posterior probability, BS: bootstrap support.

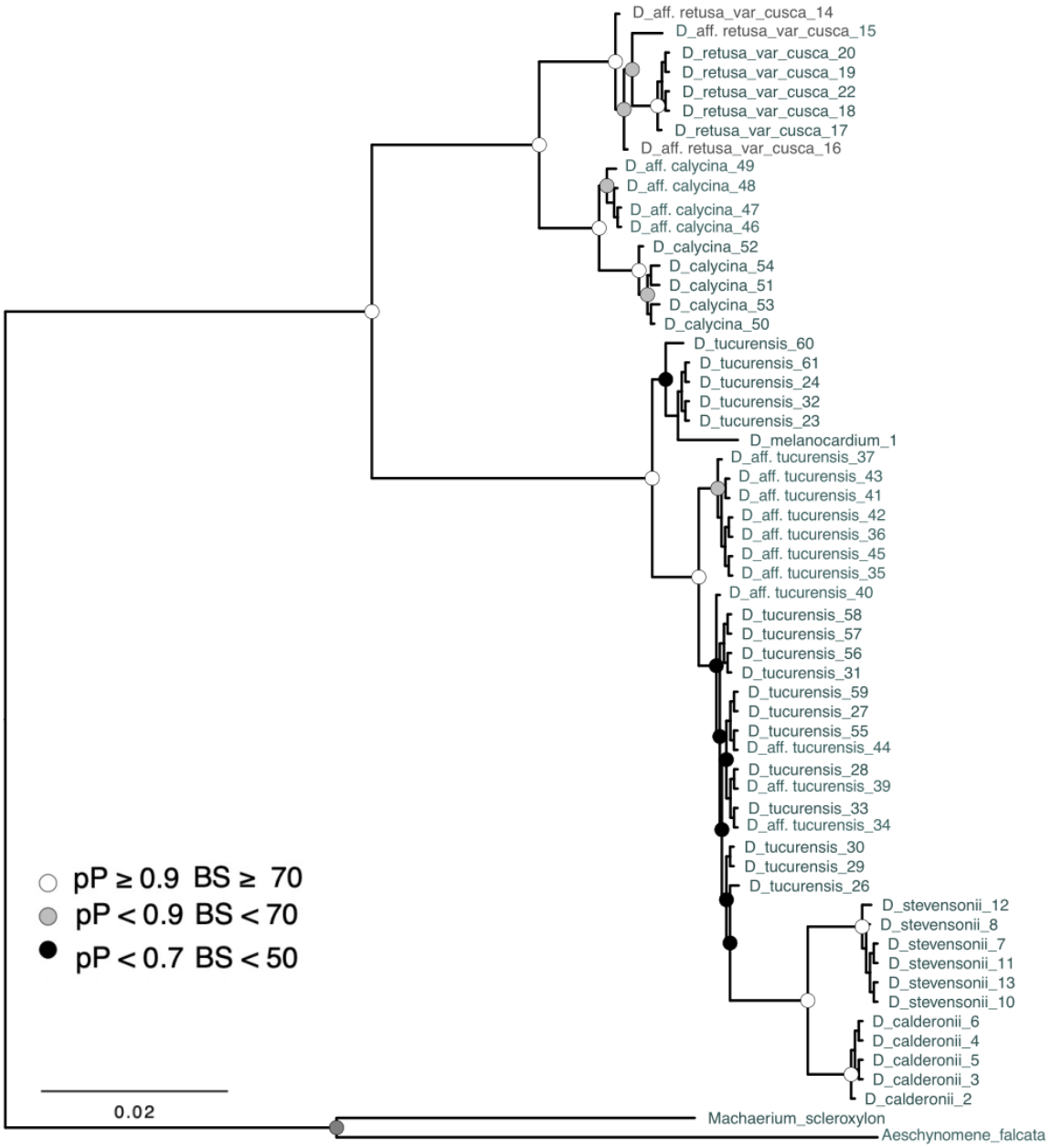

Figure S4. Phylogenetic relationships among tree species of the *Dalbergia* genus based on sequences data of concatenated loci (ITS + matK + trnH-psbA). Nodal support for BI/ML analysis is represented by the color of the circles. Nodes without circles reported BI/ML values lower than 0.7/50. Tip labels according to morphological identification. pP: posterior probability, BS: bootstrap support.

Table S1. List of species responsible for ambiguous identifications.

No.	Original label	idBM	Marker	No.	Original label	idBM	Marker
1	D_tucurensis_34	D_aff.tucurensis	I	37	D_aff.tucurensis _39	D_calderonii	M
2	D_tucurensis_35	D_aff.tucurensis	I	38	D_aff.tucurensis _40	D_calderonii	M
3	D_tucurensis_36	D_aff.tucurensis	I	39	D_aff.tucurensis _42	D_calderonii	M
4	D_tucurensis_37	D_aff.tucurensis	I	40	D_aff.tucurensis _44	D_calderonii	M
5	D_tucurensis_39	D_aff.tucurensis	I	41	D_aff.tucurensis _45	D_calderonii	M
6	D_tucurensis_40	D_aff.tucurensis	I	42	D_tucurensis_26	D_calderonii	M
7	D_tucurensis_41	D_aff.tucurensis	I	43	D_tucurensis_27	D_calderonii	M
8	D_tucurensis_42	D_aff.tucurensis	I	44	D_tucurensis_28	D_calderonii	M
9	D_tucurensis_43	D_aff.tucurensis	I	45	D_tucurensis_29	D_calderonii	M
10	D_tucurensis_44	D_aff.tucurensis	I	46	D_tucurensis_30	D_calderonii	M
11	D_tucurensis_45	D_aff.tucurensis	I	47	D_tucurensis_31	D_calderonii	M
12	D_aff.tucurensis_23	D_tucurensis	I	48	D_tucurensis_33	D_calderonii	M
13	D_ aff.tucurensis _ 24	D_tucurensis	I	49	D_tucurensis_55	D_calderonii	M
14	D_tucurensis_26	D_aff.tucurensis	I	50	D_tucurensis_56	D_calderonii	M
15	D_tucurensis_27	D_aff.tucurensis	I	51	D_tucurensis_57	D_calderonii	M
16	D_tucurensis_28	D_aff.tucurensis	I	52	D_tucurensis_58	D_calderonii	M
17	D_tucurensis_29	D_aff.tucurensis	I	53	D_tucurensis_59	D_calderonii	M
18	D_tucurensis_30	D_aff.tucurensis	I	54	D_melanocardium_1	D_tucurensis	T
19	D_tucurensis_31	D_aff.tucurensis	I	55	D_aff.retusa_var_cusca _16	D_retusa	T
20	D_ aff.tucurensis _32	D_tucurensis	I	56	D_retusa_var_cusca_17	D_unknown	T
21	D_tucurensis_33	D_aff.tucurensis	I	57	D_retusa_var_cusca_18	D_unknown	T
22	D_tucurensis_55	D_aff.tucurensis	I	58	D_retusa_var_cusca_19	D_unknown	T
23	D_tucurensis_56	D_aff.tucurensis	I	59	D_retusa_var_cusca_20	D_unknown	T
24	D_tucurensis_57	D_aff.tucurensis	I	60	D_retusa_var_cusca_21	D_unknown	T
25	D_tucurensis_58	D_aff.tucurensis	I	61	D_retusa_var_cusca_22	D_unknown	T
26	D_tucurensis_59	D_aff.tucurensis	I	62	D_ aff.retusa_var_cusca _14	D_retusa	T
27	D_ aff.tucurensis _61	D_tucurensis	I	63	D_ aff.retusa_var_cusca _15	D_retusa	T
28	D_calderonii_2	D_tucurensis	M	64	D_aff.calycina_46	D_unknown	T
29	D_calderonii_3	D_tucurensis	M	65	D_ aff.calycina _47	D_unknown	T
30	D_calderonii_4	D_tucurensis	M	66	D_ aff.calycina _48	D_unknown	T
31	D_calderonii_5	D_tucurensis	M	67	D_ aff.calycina _49	D_unknown	T
32	D_calderonii_6	D_tucurensis	M	68	D_aff.tucurensis _23	D_tucurensis	T
33	D_ aff.tucurensis _34	D_calderonii	M	69	D_aff.tucurensis _24	D_tucurensis	T
34	D_ aff.tucurensis _35	D_calderonii	M	70	D_tucurensis_25	D_sp01	T
35	D_ aff.tucurensis _36	D_calderonii	M	71	D_aff.tucurensis _32	D_tucurensis	T
36	D_ aff.tucurensis _38	D_calderonii	M	72	D_tucurensis_60	D_aff.tucurensis	T

idBM: the assignment of a species name according to the best match criterion. I: ITS, M: matK, T: trnH-psbA