

## Phylogenetic Relationships of *Tovomita* (Clusiaceae): Carpel Number and Geographic Distribution Speak Louder than Venation Pattern

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Communicating Editor: Timothy M. Evans

**Abstract**—*Tovomita* is a Neotropical clade of Clusiaceae that includes 52 species widely distributed throughout the Amazon, Atlantic, Antilles, and Chocoan/southern Mesoamerican rainforests. Species-level relationships within *Tovomita* remain largely unexplored, thus hindering our understanding of their biogeography and the evolution of key morphological characters in the genus. Here, we inferred a plastid genome phylogeny containing 18 *Tovomita* species using maximum likelihood and Bayesian inference approaches. Our results indicate that current infrageneric classification of *Tovomita*, which relies largely on leaf venation, does not reflect phylogenetic relationships. Instead, we identify carpel number as a more reliable morphological trait for infrageneric classification: clades within *Tovomita* tend to include species that possess either four or five (or more) carpels. Moreover, groups of species within *Tovomita* tend to exhibit a high degree of geographic endemism corresponding to their clade affiliation: species within these clades are restricted to either Amazon or Atlantic forests. The well supported clade of Atlantic forest inhabitants we identify is sister to a clade of mostly Amazonian species that also includes Amazon and Atlantic forest disjunct species, which are more closely related to Amazonian than to other Atlantic forest species. These findings represent a first important step in elucidating morphological evolution and biogeography in this widespread genus of neotropical rainforest trees and shrubs.

**Keywords**—Amazon, Atlantic forest, Clusioid clade, next-generation sequencing, plastome.

*Tovomita* Aubl. is the third largest genus within the family Clusiaceae Lindl. (~800 pantropical species) and has been the focus of recent taxonomic, morphological, and phylogenetic investigation (Marinho et al. 2019). Marinho et al. (2019) demonstrated that the genus was polyphyletic as traditionally circumscribed and consists of at least three distantly related clades necessitating several taxonomic changes. The largest of these clades includes members of the newly recircumscribed *Tovomita* s. s. (hereafter *Tovomita*) (Marinho et al. 2019), which comprises 52 species of dioecious trees and shrubs (Fig. 1A) and is the focus of the present study. These species are well-defined morphologically and are characterized by their reddish cortex, yellow exudate (Fig. 1B), terminal inflorescences (Fig. 1C), and floral buds enclosed by an outer pair of sepals prior to anthesis (Fig. 1D; Engler 1925; Cuello 1998, 2003; Marinho et al. 2016b, 2019). The remaining species formerly recognized in *Tovomita* sensu lato (s. l.) have been transferred to the recently described genus *Arawakia* L. Marinho [*T. weddelliana* Planch. & Triana, the *T. weddelliana* complex (sensu Gahagen et al. 2015)], and the genus *Chrysochlamys* Poepp. (*T. croatii* Maguire) (Marinho et al. 2019). Members of the newly circumscribed *Tovomita* have a broad geographical distribution in the neotropics (Fig. 2). They are nearly entirely restricted to rainforests from 1) the Amazon including the Guyana Shield (40 species), 2) the Atlantic forest of Brazil (12 species), 3) the Chocoan/southern Mesoamerican region (four species), and 4) the lower Antilles (one species; Marinho et al. 2016b, 2019). Among these, four species occur in both the

Atlantic and Amazon forests [*T. choisyana* Planch. & Triana, *T. fructipendula* (Ruiz & Pav.) Cambess., *T. guianensis* Aubl., and *T. mangle* G. Mariz], or are widespread throughout three centres of diversity [*T. longifolia* (Rich.) Hochr. occurs in all regions except the lower Antilles].

Current infrageneric classification of the more broadly circumscribed *Tovomita* was established by Vesque (1893), who recognized two sections in the genus: *Tovomita* sect. *Clusiifoliae* Vesque, characterized by leaves with numerous secondary veins not arcuate near the blade margin (Fig. 1E), and *T.* sect. *Chrysochlamydifoliae* Vesque, characterized by few secondary veins conspicuously arcuate near the blade margin (Fig. 1F). Engler (1923) subsequently combined these two sections and placed them as subsections of *Tovomita* sect. “*Eutovomita*” Engl., which was proposed to accommodate species with terminal inflorescences. He also proposed a section to accommodate species with axillary inflorescences (*T.* sect. *Dystovomita* Engl.). D’Arcy (1978) subsequently elevated *T.* sect. *Dystovomita* to generic rank, and maintained two subsections in “*Eutovomita*”, which are now informally treated as *Clusiifoliae* and *Chrysochlamydifoliae* within *Tovomita* (Mariz 1974; Barros and Mariz 1982; Marinho et al. 2015a, 2015b, 2016a). Eighteen species of *Clusiifoliae* were transferred to *Arawakia*, and one species of *Chrysochlamydifoliae* was transferred to *Chrysochlamys* to accommodate recent phylogenetic discoveries (Marinho et al. 2019). However, a more expansive phylogenetic framework for *Tovomita* is lacking and hinders our ability to devise a useful infrageneric classification.



FIG. 1. Diagnostic features of *Tovomita* species. A. Arboreal habit of *T. fructipendula* from the Amazon forest. B. Slashed trunk, showing reddish cortex and yellow latex (arrows) in *T. fructipendula*. C. Terminal inflorescence of *T. mangle*. D. Floral bud of *T. megantha* L. Marinho & Amorim. E. *T. calophyllophylla* García-Villacorta & Hammel, leaf venation pattern from the Clusiifoliae informal group (= *T.* subsect. *Clusiifoliae* sensu Vesque [1893]) (lower left symbol as in Fig. 3). F. *T. auriculata* Cuello, leaf venation pattern from the Chrysochlamydifoliae informal group (= *T.* subsect. *Chrysochlamydifoliae* sensu Vesque [1893]) (lower left symbol as in Fig. 3). Photos: A, B, D, and E by L. Marinho; C by A. Amorim; F by L. Torres Montenegro.

This is especially problematic because the current classification suffers from single character taxonomy, namely the reliance on venation patterns, which may not reflect the phylogeny.

Here, we investigate the phylogeny of *Tovomita* to test whether the current infrageneric classification is supported by molecular data. We additionally explore the taxonomic utility of morphological characters and geographic distribution.

#### MATERIALS AND METHODS

**Taxonomic Sampling**—Plastome data were obtained for 24 accessions, including three outgroup taxa [*Clusia polysepala* Engl., *C. renggerioides* Planch. & Triana, and *Dystovomita paniculata* (Donn. Sm.) Hammel]. The remaining twenty-one accessions represent 18 species of *Tovomita*, which is approximately 34% of the validly published species within the genus. We took care to sample widely across their geographic distribution, including Amazon, Atlantic forest, and the Chocó/southern Mesoamerica. In addition, we scored the following morphological characters for each specimen of *Tovomita* we sampled: carpel number [4-carpellate vs. 5–6(–7)-carpellate flowers], leaf venation (Clusiifoliae vs. Chrysochlamydifoliae types, see Fig. 1), and biogeographic distribution (Amazon, Atlantic forest, Chocóan/southern Mesoamerica region, and lower Antilles). We also included three species that have wider ranges and are disjunct between Amazon and Atlantic forests (*T. choisyana* Planch. & Triana, *T. fructipendula* (Ruiz & Pav.) Cambess., and *T. mangle* G. Mariz). Among these, *T. fructipendula* was sampled from both areas of their disjunct range. Field-collected specimens were deposited at the CEPEC and HUEFS herbaria (acronyms follow Thiers 2020). A list of voucher and associated NCBI accessions is provided in Appendix 1.

**DNA Isolation, Amplification, and Sequencing**—We isolated total genomic DNA from 0.01 g of silica gel dried leaf or herbarium specimens using the Maxwell® 16 tissue DBA purification kit (Promega Corporation, Inc., Madison, Wisconsin). Additional DNA samples were obtained from herbarium specimens deposited at AAU, INPA, and NY (Appendix 1; herbarium acronyms following Thiers [2020]). DNA from *Dystovomita paniculata* and *T. longifolia* (Rich.) Hochr. were extracted using the CTAB method (Doyle and Doyle 1987). Genomic libraries were prepared with ca. 70 ng of genomic DNA, indexed for Illumina multiplex sequencing with the Kapa HyperPlus library prep kit (Kapa Biosystems, Inc., Massachusetts) with Nextflex-Ht barcodes (Bioo Scientific Corporation, Texas). Libraries were fragmented to 350–400 base pairs (bp) and their quality, expected size, and concentration were verified with the Agilent TapeStation 2200 (Agilent Technologies, Inc., Waldbronn, Germany) and the Qubit dsDNA HS Assay Kit on a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, California). All 24 libraries were diluted to 0.7 nM, pooled and sequenced with the Illumina Hi-Seq 2 × 125 on the Genome Analyzer II (Illumina, Inc., San Diego, California) at the Bauer Core Sequencing Facility (<https://bauercore.fas.harvard.edu/>). These protocols largely follow Marinho et al. (2019).

**Plastid Genome Assembly and Annotation**—We applied a reference-guided strategy to assemble plastid genomes using Geneious 9.0.5 (Kearse et al. 2012). The plastid genome of *Manihot esculenta* Crantz (Euphorbiaceae, NCBI's Reference Sequence: NC\_009143.1), including both inverted repeat regions, was used as reference. Reads were trimmed prior to assembly based on the maximum 5% error probability. We also applied the successive reference approach to obtain higher quality assemblies as specified by Zhang et al. (2015) and recently tested by Marinho et al. (2019) in Clusiaceae. Reads were subsequently aligned to the reference sequence and consensus sequences were called to match at least 50% of the aligned reads. When necessary we used the standard symbols proposed by Cornish-Bowden (1985) to represent ambiguous sites. Regions with less than 2 × read coverage were masked to produce the final assembly. Assemblies were annotated using the BLAST-like transfer annotation tool in Geneious based on the *Manihot esculenta* annotation. An identity cut-off of 60% was applied. We removed low-quality assemblies with less than 5000 bp unambiguous sites from subsequent analyses.

**Plastid Genome Alignment and Phylogenetic Inference**—We used MAFFT v. 7.299b (Katoh and Standley 2013) to align plastid genomes, applying the fast Fourier transformation approximation option, with partition size set to 1000, and three iterative refinements. The aligned sequences were prepared for phylogenetic analyses by removing sites with > 70% missing data using trimAL 1.4.rev15 (Capella-Gutiérrez et al. 2009). The best partition scheme for maximum likelihood (ML) analysis applying the GTRGAMMA model was identified by PartitionFinder 2 using the heuristic search algorithm 'rcluster' (Lanfear et al. 2012). We inferred an ML phylogeny of the 24 species using RAxML v. 8.2.4 (Stamatakis 2014) with 1000 rapid bootstrap replicates followed by a thorough ML search (-f a -N 1000). Bayesian inference (BI) was performed using



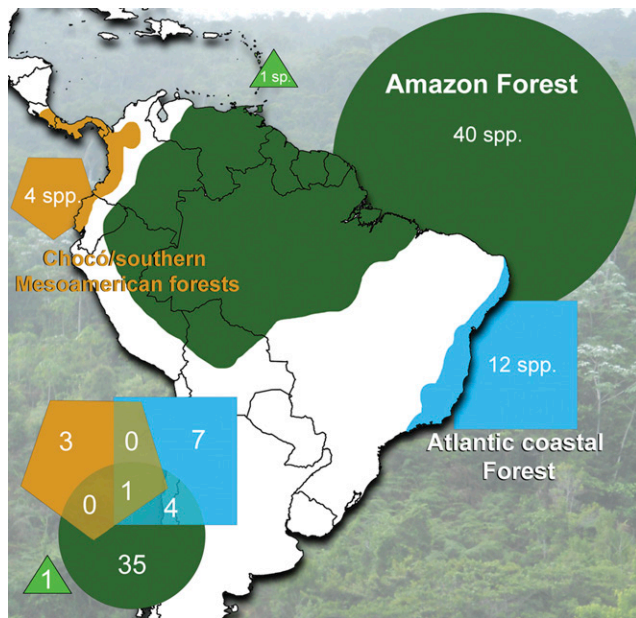


FIG. 2. Distribution of *Tovomita* species across the rainforested regions of the American tropics. The Venn diagram presents the number of exclusive and disjunct species.

PhyloBayes MPI 1.7a (Lartillot et al. 2013) under the CAT-GTR model (Lartillot and Philippe 2004), which accounts for across-site rate heterogeneity using an infinite mixture model. Two independent Markov Chain Monte Carlo (MCMC) analyses were conducted for each concatenated nucleotide matrix. Stationarity from both MCMC analyses were determined using Tracer 1.5. We ran each MCMC analysis until the minimum effective sampling size estimated by Tracer exceeded 200 for all parameters in each chain. This yielded 73,241 and 37,293 sampled trees for each run. The largest discrepancy observed across all bipartitions was 0.10, indicating convergence of the two independent runs. Bayesian posterior consensus trees and parameter estimates were calculated using the 'bcomp' option in PhyloBayes using a burn-in of > 10%, and subsampling every 10 trees.

## RESULTS

The BI and ML analyses recovered a monophyletic *Tovomita* with strong support (100 bootstrap percentage [BP] / 1.0 posterior probability [PP], Fig. 3). The ML analysis recovered two main clades. Clade A (94 BP) includes two species, the Amazonian endemic *T. volkeri* L. Marinho plus the Amazon/Atlantic forest disjunct species *T. fructipendula*. Clade B (96 BP) includes three well-supported subclades: *T. calophyllophylla* + *T. stylosa* Hemsl. (100 BP), clade C (99 BP), or the "Atlantic forest clade," and clade D. Clade D (99 BP) was fully resolved and includes the Mesomeric representative *T. longifolia*, which is the only species spanning three of the four main areas inhabited by *Tovomita* species (see Fig. 2). Members of clade D include mostly Amazon forest endemics plus disjunct species between Amazon and Atlantic forests (i.e. *T. choisyana* and *T. mangle*).

The BI and ML analyses produced largely congruent results but disagreed with regard to the position of *T. longifolia* and *T. salimena* L. Marinho & Amorim (Fig. 3). The BI analysis recovered a polytomy at the base of the tree, including a strongly supported clade A (0.97 PP), a weakly supported clade B (0.67 PP), and *T. longifolia*. Within clade C, *T. salimena* was highly supported as sister to *T. leucantha* (Schltdl.) Planch.

& Triana (1.0 PP). Clade D includes a polytomy of three clades: *T. acutiflora* M.S. Barros & G. Mariz, *T. choisyana* plus *T. mangle* (0.88 PP), and a weakly supported clade (0.65 PP) including five Amazonian endemic species.

Finally, our sampling of key characters suggests that carpel number and the biogeographic origin, but not leaf venation, are likely important in delimiting groups of species groups in *Tovomita*.

## DISCUSSION

The plastome phylogeny presented here includes ~34% of the currently recognized species of *Tovomita* and greatly clarifies interspecific relationships within the genus. It also reveals three key findings related to the morphology and geographical distribution of the group. First, there is no support for the current infrageneric classification of *Tovomita*, which is mainly based on patterns of leaf venation. Second, carpel number appears to be more phylogenetically informative for delimiting natural species groups within the genus. Third, the clades recovered within *Tovomita* exhibit strong biogeographic endemism. Here, species endemic to the Atlantic forest formed a well-supported clade that is nested within a grade of species primarily restricted to Amazonian forest.

**Non-monophyly of Traditional Infrageneric Groups**—Despite the taxonomic utility of leaf venation for delimiting individual species of *Tovomita*, higher order groupings by Vesque (1893) and Engler (1923) that utilize this feature are not reflective of monophyly. Vesque (1893) first proposed an infrageneric classification for *Tovomita* based on the arrangement of secondary and intersecondary leaf veins (Fig. 1E–F). His *T. sect. Clusiifoliae* comprised species with several closely spaced and similarly gauged secondary and intersecondary veins, which do not arch near the leaf blade margin (Fig. 1E). In contrast, species of *T. sect. Chrysochlamydidifoliae* have fewer secondary veins, which arch near the margin. The intersecondary veins are also more widely spaced and thinner than the secondary ones (Fig. 1F). This classification has been extensively used and discussed in previous taxonomic and morphological investigations of the group (e.g. Barros 1979; Marinho et al. 2015a, 2015b, 2016a, 2016c). Our phylogeny clearly demonstrates that leaf venation should not be utilized to delimit higher order groups in *Tovomita*.

**Systematic Utility of Carpel Number in *Tovomita***—Our phylogeny suggests that carpel number better reflects phylogenetic relationships within *Tovomita* than leaf venation. Most species of *Tovomita* (~72%) have 4-carpellate flowers, with their fruits splitting into four valves as they ripen. The remaining species (~28%) bear 5–6(–7)-carpellate flowers, such as *T. gazelii* Poncy & Offroy and *T. laurina* Planch. & Triana, where variation in carpel number (five to seven carpels) can be observed within a single population (Engels and Marinho 2018). Members of clade A are exclusively 4-carpellate; species in clade B possess mostly four carpels, and 5(–7)-carpellate species in this clade likely evolved independently at least three times (Fig. 3).

The directional evolution of this trait is yet unclear. Because *Dystovomita* (Engl.) D'Arcy and *Tovomitopsis* Planch. & Triana are both 4-carpellate, and form a polytomy with (*Tovomita* (*Arawakia* (*Chrysochlamys*, *Clusia*))) (Marinho et al. 2019), it is likely that 5-carpellate species of *Tovomita* are derived from 4-carpellate ancestors. A deeper investigation including

ancestral reconstruction of carpel number within Clusiaceae, with expanded taxon sampling, would be required to appropriately assess the nature of these character changes in the tribe. Nevertheless, our results highlight that species from some clades of *Tovomita* share the same carpel number, thus

demonstrating the taxonomic utility of this character in the genus.

**Geographic Endemicity of *Tovomita***—Clades within *Tovomita* exhibit a moderate degree of geographic endemicity, indicating that geographical distribution may have

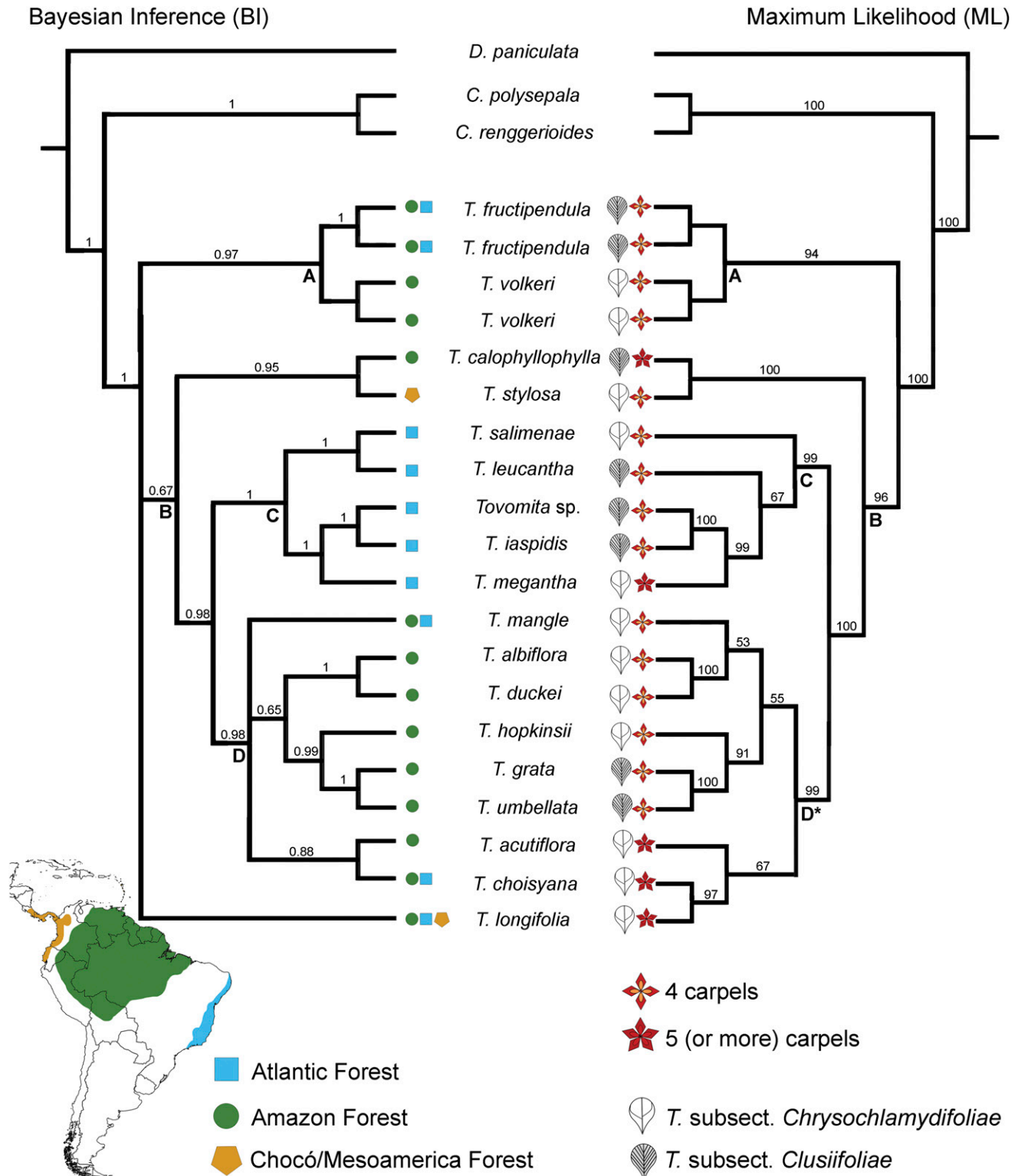


FIG. 3. Plastid genome trees of species of *Tovomita*. Support values are indicated above branches as follows: BI posterior probabilities (PP) and ML bootstrap percentage (BP). Branches with support values below 50% BP and 0.50 PP are collapsed. Clades A–D are discussed in the text. The asterisk in clade D of ML analysis indicates that clades D of ML and BI are not composed of the same species due to the absence of *Tovomita longifolia* in clade D of ML.

systematic utility. We identify that species endemic to Atlantic forest form a clade (clade C, Fig. 3) that is nested within a grade of species primarily restricted to Amazonian forest. In addition to their biogeographical affinities, members of the Atlantic forest clade also possess a unique combination of features, including young leaves with a

reddish petiole (Fig. 4A), dichasial inflorescences with up to nine flowers (Fig. 4B), sessile stigmas (Fig. 4C), and fruits with a smooth epicarp (Fig. 4D).

We sampled four species that exhibit disjunct distributions in Amazon and Atlantic forests. These species are mostly distributed from the northeastern coast of Brazil to relictual

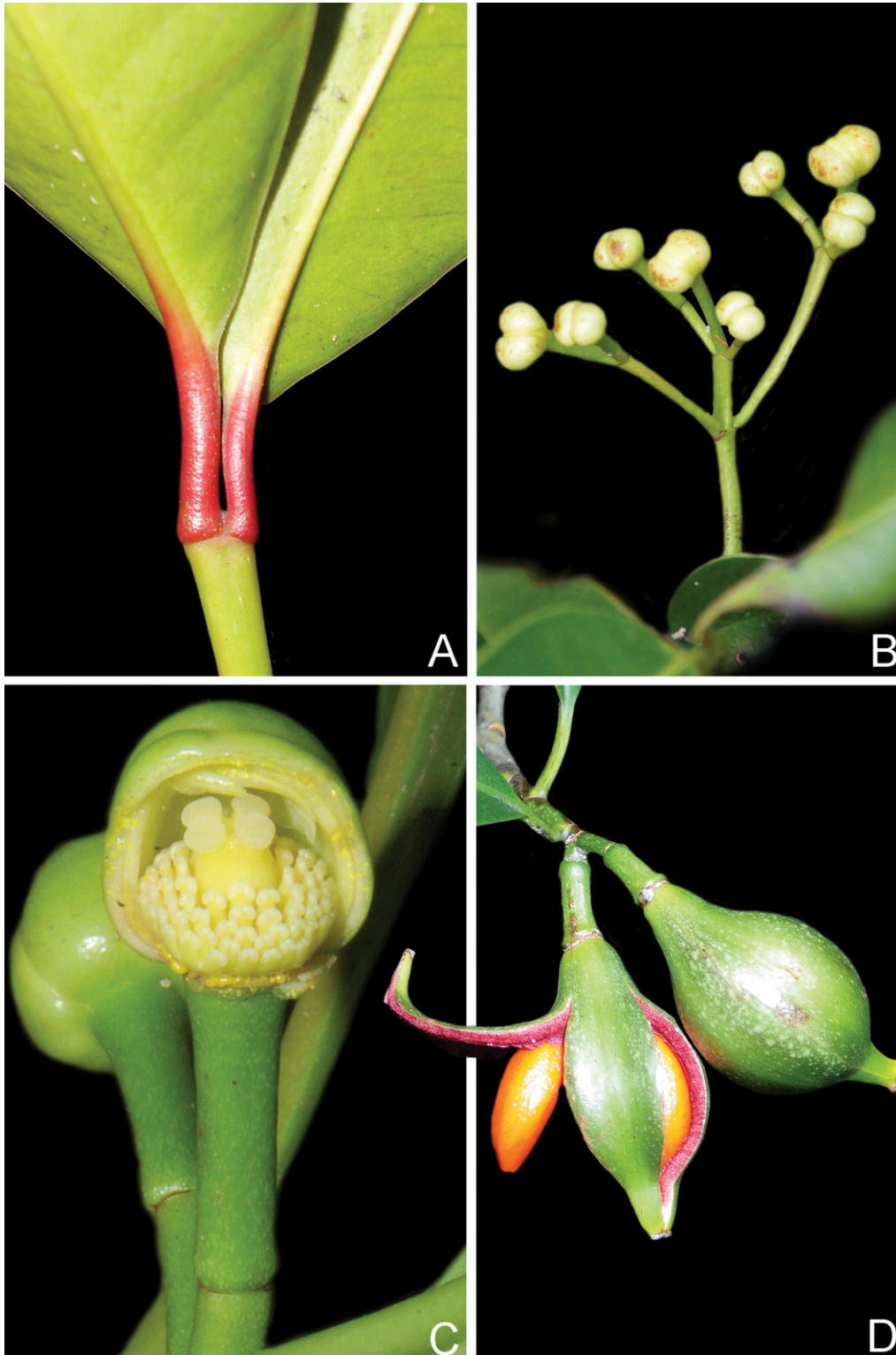


FIG. 4. Diagnostic features of Atlantic clade species. A. Reddish petiole in *Tovomita glazioviana*. B. Thyrsoid inflorescence in *T. iaspidis*. C. Sessile stigmas in *T. leucantha*. D. Smooth epicarp in *T. riedeliana*. Photos: A by L. Marinho; B by A. Amorim; C by A. Brandão; D by L. Nusbaumer.

rainforest enclaves dispersed amongst the seasonally dry Caatinga in the states of Ceará and Maranhão (i.e. *T. mangle* [Marinho 2020]). Three of those disjunct species (*T. choisyana*, *T. longifolia*, and *T. mangle*) are nested with high support within an Amazonian clade of *Tovomita* (Fig. 3) in the ML analyses, suggesting that Atlantic forest populations might have been derived from Amazonian source populations. This and other biogeographic hypotheses, however, require tests based on more comprehensive taxonomic and geographic sampling, and a timeframe built upon reliable molecular dating to better understand the biogeographic history of *Tovomita* in the Neotropics.

Our results also suggest at least two independent dispersals to the Chococoan/southern Mesoamerica region, as *T. stylosa* and *T. longifolia*, the latter represented here by a specimen from Costa Rica, are not closely related. The phylogenetic distance between these species should be interpreted cautiously because there is morphological evidence that *T. longifolia* may not be monophyletic, and may instead represent three species (Marinho pers. obs.). The question whether Chococoan/southern Mesoamerican *Tovomita* is a well-supported monophyletic group could be tested by expanding taxon sampling to include representatives of *T. morii* Maguire, *T. plumieri* Griseb., and *T. trojitana* Cuatrec. in subsequent phylogenetic studies.

Our study is the first to clarify more fine-scale phylogenetic relationships within *Tovomita* and to evaluate morphological evolution and biogeography within the genus. Because the type species of the genus, *T. guianensis* Aubl., could not be sampled, we refrain from proposing a formal infrageneric classification to *Tovomita*. Despite this, we demonstrate that current infrageneric classification of *Tovomita*, which has relied largely on leaf venation pattern, does not reflect phylogenetic relationships. Instead, we identify that carpel number and geographical distribution, previously ignored for infrageneric circumscriptions, are more reliable for a phylogenetic classification of the genus.

#### ACKNOWLEDGMENTS

The authors thank the following herbaria for providing leaf tissues: AAU, CEPEC, HUEFS, INPA, NY, and RB (abbreviations follow Thiers 2020). Funding for this research was provided by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil) by Ph.D. fellowships awarded to LCM (# 141561/2015-7) and MFF (# 141560/2015-0), and Research Productivity fellowships to AMA (# 312404/2018-2) and PF (# 310502/2019-5). We acknowledge additional funding provided to CCD from Harvard University. LCM was also supported in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (PDSE Capes, # 88881.135403/2016-01), Harvard University (HUH Travel Grant awarded in 2017), and the International Association for Plant Taxonomy (IAPT Grant awarded in 2016). We thank our colleagues in the Davis lab and other members in the Department of Organismic and Evolutionary Biology at Harvard University for technical assistance and valuable discussions. We also thank members of LCM's Ph.D. dissertation committee for their valuable comments.

#### AUTHOR CONTRIBUTIONS

LCM, AMA, PF, and CCD conceived the project. LCM and AMA contributed tissue samples. LCM and XD collected DNA sequences. LCM and LC performed the experiments. LCM, PF, CCD, and MFF analyzed the data and wrote the paper with comments and editing from all other co-authors. LCM prepared the figures.

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APPENDIX 1. List of Clusiaceae taxa included in this study. Taxon, country of origin, largest political subdivision if available, collector(s) and collection number (herbarium code, if available), GenBank accession number of plastome, if available.

Outgroup: *Clusia polysepala* Engl., Brazil, Pará, L.C. Marinho 1305 (CEPEC), SAMN09652122. *C. renggerioides* Planch. & Triana, Brazil, Amazonas, L.C. Marinho 1376 (HUEFS), SAMN09652123. *Dystovomita paniculata* (Donn.Sm.) Hammel, Costa Rica, B.E. Hammel 25295, SAMN09652124. Ingroup: *T. acutiflora* M.S.Barros & G.Mariz, Brazil, Amazonas, L.C. Marinho 1370 (HUEFS), SAMN09652127. *T. albiflora* A.C.Sm., Venezuela, Bolívar, J.A. Steyermark 75192 (NY), SAMN15399397. *T. calophyllophylla* García-Villacorta & Hammel, Peru, J. Vormisto 579

(AAU), SAMN15399398. *T. choisyana* Planch. & Triana, Brazil, Bahia, L.C. Marinho 460 (CEPEC), SAMN09652129. *T. duckei* Huber, Bolivia, La Paz, B.A. Krukoff 11043 (NY), SAMN15399399. *T. fructipendula* (Ruiz & Pav.) Cambess., Brazil, Bahia, L.C. Marinho 950 (CEPEC), SAMN09652131. *T. fructipendula* (Ruiz & Pav.) Cambess., Brazil, Pará, L.C. Marinho 1241 (CEPEC), SAMN15399400. *T. grata* Sandwith, Brazil, Amazonas, L.C. Marinho 1011 (CEPEC), SAMN15399401. *T. hopkinsii* Bittrich & L.Marinho, Brazil, Amazonas, C.A. Sothers 452 (INPA), SAMN09652134. *T. iaspidis* L.Marinho & Amorim, Brazil, Bahia, L.C. Marinho 620 (CEPEC), SAMN15399402. *T. leucantha* (Schltdl.) Planch. & Triana, Brazil, Rio de Janeiro, L.C. Marinho 888 (RB), SAMN09652136. *T. longifolia* (Rich.) Hochr., Costa Rica, R. Aguilar 12290, SAMN09652137. *T. mangle* G.Mariz, Brazil, Bahia, R. Barbosa-Silva 684 (CEPEC), SAMN15399403. *T. megalantha* L.Marinho & Amorim, Brazil, Bahia, A.M. Amorim 9097 (CEPEC), SAMN15399404. *T. salimena* L.Marinho & Amorim, Brazil, Minas Gerais, L.C. Marinho 981 (HUEFS), SAMN15399405. *T. stylosa* Hemsl., Colombia, Vaupés, A. Gentry 56960 (NY), SAMN15399406. *T. umbellata* Benth., Brazil, Amazonas, L.C. Marinho 1345 (HUEFS), SAMN09652139. *T. volkeri* L.Marinho, Brazil, Amazonas, L.C. Marinho 1021 (CEPEC), SAMN15399407. *T. volkeri* L.Marinho, Brazil, Amazonas, L.C. Marinho 1365 (HUEFS), SAMN15399408. *Tovomita* sp., Brazil, Bahia, L.C. Marinho 834 (CEPEC), SAMN15399409.