Admixture may be extensive among hyperdominant Amazon rainforest tree species

Drew A. Larson[1]* https://orcid.org/0000-0002-7557-9999, Oscar M. Vargas[2] https://orcid.org/0000-0002-5654-5873, Alberto Vicentini[3] https://orcid.org/0000-0002-5906-9358 and Christopher W. Dick[1,4] https://orcid.org/0000-0001-8745-9137

[1]Department of Ecology & Evolutionary Biology, University of Michigan, Ann Arbor, MI, 48109, USA
[2]Department of Biological Sciences, Humboldt State University, Arcata, CA, 95521, USA
[3]Instituto Nacional de Pesquisas da Amazônia (INPA), CEP 69067-375, Manaus, AM, Brazil
[4]Smithsonian Tropical Research Institute, Panama City, Republic of Panama

*Author for correspondence: Drew A. Larson
Email: larsonda@umich.edu
Tel: +1 (320) 894 0086

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Summary

- Admixture is a mechanism by which species of long-lived plants may acquire novel alleles. However, the potential role of admixture in the origin and maintenance of tropical plant diversity is unclear. We ask whether admixture occurs in an ecologically important clade of *Eschweilera* (Parvifolia clade, Lecythidaceae), which includes some of the most widespread and abundant tree species in Amazonian forests.

- Using target capture sequencing, we conducted a detailed phylogenomic investigation of 33 species in the Parvifolia clade and investigated specific hypotheses of admixture within a robust phylogenetic framework.

- We found strong evidence of admixture among three ecologically dominant species, *E. coriacea*, *E. wachenheimii*, and *E. parviflora*, but a lack of evidence for admixture among other lineages.

Accepted species were largely distinguishable from one another, as was geographic structure within species.

- We show that hybridization may play a role in the evolution of the most widespread and ecologically variable Amazonian tree species. While admixture occurs among some species of *Eschweilera*, it has not led to widespread erosion of most species' genetic or morphological identities. Therefore, current morphological based species circumscriptions appear to provide a useful characterization of the clade's lineage diversity.

Keywords: Amazon basin; hyperdominance; adaptive introgression; hybridization; Parvifolia clade; Lecythidaceae (Brazil nut family); target enrichment sequencing; tropical diversity

Introduction

The extent to which hybridization and introgression (i.e. admixture) have affected the evolutionary history of tropical trees are only beginning to be understood. Admixture is expected to have various evolutionary consequences depending on the context of its occurrence, ranging from infrequent, localized production of hybrid offspring to the formation of new species (Rieseberg & Wendel, 1993). Adaptive introgression is a possible mechanism by which tropical tree populations may acquire favorable alleles, as has been demonstrated in various other plant clades (e.g. Martin *et al.*, 2006; Whitney *et al.*, 2010; Pease *et al.*, 2016; Leroy *et al.*, 2020), and may facilitate local adaptation beyond what might occur through selection acting on standing genetic variation and *de novo* mutations (Suarez-Gonzalez *et al.*, 2018).

Hybridization amongst tropical trees has historically been considered a relatively rare phenomenon, primarily because of the dearth of morphological intermediates in herbarium specimens of tropical tree floras (Ashton, 1969; Parnell *et al*., 2013). However, recent work using next generation sequencing methods has demonstrated evidence of hybridization in tropical trees including in *Brownea* (Fabaceae; Schley *et al.*, 2020), *Diosypyros* (Ebenaceae; Linan *et al.*, 2020), *Melicope* (Rutaceae; Paetzold *et al*., 2019) and *Metrosideros* (Myrtaceae; Choi *et al.*, 2020). Caron *et al.* (2019) found that across tree taxa at a site in northern French Guiana, chloroplast haplotype diversity was more frequent in

species with a local congener than those without, which they attribute to introgression. However, direct evidence of hybridization remains elusive for most clades of tropical trees. Because tests for admixture are inherently comparative, tests for admixture should ideally be nested within a robust and broadly inclusive phylogeny (Eaton *et al.*, 2015). Such phylogenies are not yet available for many tropical clades, though phylogenomic datasets are becoming increasingly available (e.g. Prata *et al.*, 2018; Couvreur *et al.*, 2019; Loiseau *et al.*, 2019; Linan *et al*., 2020; Christe *et al*., 2021). Investigations that characterize gene flow at well-studied forest plots may also enhance our understanding of the role of admixture in tropical forests, because 1) gene pools can be delimited without having to consider the confounding effects of geographic variation (Linan *et al*., 2020; Schley *et al*., 2020), and 2) permanently tagged trees provide a kind of "living herbarium" in which variation in field characters not evident in herbarium collections (e.g. branching architecture, microhabitat preferences, tree size) may be studied.

Target capture sequencing, also called target enrichment, is becoming increasingly popular for phylogenomic studies of non-model plants (Cronn *et al.*, 2012; Baker *et al*., 2021) and often produces datasets with low missing data, even when the input DNA is partially degraded. The sizes of target loci vary, but generally range from hundreds to a few thousand base pairs (bp) in length. The number of targets also varies, but is frequently a few hundred loci, which is usually sufficient for phylogeny reconstruction but is far fewer than is typically used for inferring admixture, especially compared to methods such as RADseq, which can recover tens of thousands of RAD loci (Eaton & Ree, 2013; Eaton *et al.*, 2015; Johnson *et al.*, 2018; Vargas *et al.*, 2020). Gene tree-based methods for inferring admixture using species networks can be used with several types of data, including target capture, though the resulting networks can include patterns of reticulate evolution that are sensitive to model parameters and gene tree quality (Morales-Briones *et al.*, 2020). Given this and the increasing use of target capture for studies of plant evolution comes the need to explore additional methods capable of identifying evidence of admixture.

Our study taxa are tree species in the Brazil nut family, Lecythidaceae (Ericales). Lecythidaceae are ecologically important in many Neotropical forests and several species in the genus *Eschweilera* are among the most abundant trees across the Amazon basin (ter Steege *et al*., 2013). The Parvifolia clade of *Eschweilera* comprises 66 described species, characterized by morphological features including a distinctive double-coiled androecium (Fig. 1D) and lateral arils on their seeds (Mori *et al.*, 2010 onward; Huang *et al.*, 2015). Several members of the Parvifolia clade have been described as hyperdominant (i.e. species with disproportionate abundance across a large area of the Amazon; ter Steege *et al*., 2013). The most abundant species of Lecythidaceae, *Eschweilera coriacea* (DC.) S.A.Mori, ranks third in abundance out of the more than 16,000 estimated Amazonian tree species. It is ecologically variable, thriving in floodplains as well as upland *terra firme* (Mori *et al*., 2010 onward), and is the only tree species that

attains ecological dominance in all geographic subregions of the Amazon basin (ter Steege *et al.*, 2013, 2020).

As is the case for many clades of tropical trees, species boundaries in Lecythidaceae are not precisely understood, though the taxonomy of the family is relatively well studied (e.g. Prance & Mori, 1979; Mori & Prance, 1990; Mori *et al*., 2010 onward). Previous studies have found discordance between morphology and plastid-based phylogenies, suggesting that chloroplast capture (i.e. the chloroplast of one species being introgressed into another) may be common in the group (Huang *et al.*, 2015). However, hybridization followed by repeated directional backcrossing can result in chloroplast capture with little genetic or morphological evidence of nuclear admixture (Rieseberg & Soltis, 1991). A recent study using microsatellite DNA markers suggested that the nuclear genomes of *Eschweilera* may also conflict with morphological based species circumscriptions (Heuertz *et al.*, 2020), though we are not aware of any previous studies that have shown explicit evidence of nuclear admixture in Lecythidaceae.

We addressed the following questions: 1) is there evidence of nuclear admixture among species of the Parvifolia clade of *Eschweilera*, including species that are among the most abundant and ecologically variable trees in the Neotropics? and 2) to what extent do accepted species of *Eschweilera* represent monophyletic lineages that are distinguishable from one another using nuclear genomic data? The answers to these questions may shed light on whether the hyperabundance of widespread species like *E. coriacea* could be partly explained by a history of genetic introgression. We employed a multi-faceted sampling strategy and used target capture sequencing to generate the largest phylogenomic dataset for the family to date. Our methods included the implementation of an explicit test for admixture suitable for target capture data, which may prove useful for other phylogenomic datasets.

Materials and Methods

***Focal study site and sampling strategy*—**We conducted sampling using two approaches. First, we sampled 12 focal species of the Parvifolia clade (Table 1) that co-occur at a single 100-ha forest plot in which all individuals of Lecythidaceae ≥ 10 cm diameter at 1.3m height have been tagged and identified by specialists beginning in the late 1980s (See supporting information; Notes S1; Mori & Lepsch-Cunha, 1995; Mori *et al.*, 2001). The "Lecythidaceae plot" lies within Reserve 1501, also known as Km 41, of the Biological Dynamics of Forest Fragments Project (BDFFP) located approximately 70 km north of Manaus, Brazil (2° 24' 54" S, 59° 50' 39" W). The plot was established to study the Lecythidaceae of the central Amazon, a geographic center of diversity for the clade, but an area in which its taxonomy was poorly characterized (Mori & Lepsch-Cunha, 1995). By pairing ecological studies with alpha taxonomy, the investigators sought to characterize nuanced morphological differences among species across population samples and, in doing so, identify new species and their ecological differences

(Mori & Lepsch-Cunha, 1995; Mori *et al.*, 2001). Flowers and fruits are produced only sporadically in many species of Lecythidaceae, but species determinations for each tree in the plot were made using fertile material whenever possible (Mori *et al.*, 2001). The site was re-censused in 2019, which showed there to be 6741 trees from 36 described species of Lecythidaceae (T. Milton *et al*., unpublished). Herein, we refer to this 100-ha Lecythidaceae plot as Reserve 1501.

We chose focal species that were among the most abundant and most closely related species of Lecythidaceae at Reserve 1501 (Mori & Lepsch-Cunha, 1995; Huang *et al.*, 2015). Whenever possible, we sampled four to six tagged trees of each focal species and observed a minimum of at least 100 meters between conspecifics to reduce the chances of sampling immediate relatives. Our field collections relied on prior tree identifications of S. Mori and coworkers and we prioritized collection of three individual trees that seemed to have intermediate morphology, including in branching architecture (Notes S2). For each field-collected sample, leaf tissue was desiccated in silica gel and a voucher was deposited at the BDFFP collection at the National Institute of Amazonian Research (INPA), in Manaus, Brazil. In total, our sampling included 60 individuals collected at Reserve 1501 that were identified as a focal species or suspected hybrid based on morphology (Table 1).

Our second sampling approach aimed for wider phylogenetic and geographic breadth and used herbarium material and existing forest inventory vouchers. For this broader sampling, the New York Botanical Garden Herbarium (NY) provided about half of our samples, which also included several non-focal species collected at Reserve 1501 and the surrounding area. Our overall sampling included 240 individuals from 127 of the 230 described species of Neotropical Lecythidaceae and seven outgroup species from Paleotropical genera. This included 109 individuals of the Parvifolia clade from 33 described species as well as several species that have not yet been formally described (Table S1). A full analysis of the relationships among all major clades, as well as a revised taxonomy of Lecythidaceae utilizing this sampling is forthcoming (O. Vargas *et al*., unpublished).

***Sequencing and assembly*—**We performed DNA extractions using the NucleoSpin Plant Mini Kit II (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol, but we extended the digestion step to one hour and added 5 uL of proteinase K (20 mg/mL; Qiagen, Hilden, Germany). Preparation of unenriched libraries for genome skimming and target-enriched libraries followed by 150 bp paired-end sequencing on an Illumina HiSeq4000 machine (Illumina Inc., San Diego California, USA) was performed by Rapid Genomics (Gainesville, Florida, USA). The probes used to enrich libraries were designed to capture 344 nuclear genes previously inferred to be low or single copy and genetically variable in Lecythidaceae (Vargas *et al.*, 2019). Raw reads were processed with SeqyClean (Zhbannikov *et al.*, 2017) to trim sequencing adapters, filter out low-quality reads, and trim read sections with a Phred

score < 20 using a window of 10 bp. Trimmed reads were checked with FASTQC v0.11.3 (Andrews, 2010). Target loci were assembled using HybPiper v1.3.1 (Johnson *et al.*, 2016) with default settings and a target file that included DNA sequences based on complete cDNA targets (Vargas *et al.*, 2019). The Hybpiper pipeline uses Exonerate (Slater & Birney, 2005), BLAST+ (Camacho *et al*., 2009), Biopython (Cock *et al*., 2009), BWA (Li & Durbin, 2009), SAMtools (Li *et al*., 2009), GNU Parallel (Tange, 2011), and SPAdes (Bankevich *et al*., 2012).

***Paralog filtering and alignment*—**When employing target capture, paralogs can be enriched during library preparation and recovered in locus assemblies. While evidence suggests all or most Lecythidaceae are diploid (Heuertz *et al.*, 2020), the lineage is thought to have experienced a whole genome duplication that occurred near the time of the most recent common ancestor of Ericales (Larson *et al.*, 2020). Given that paralogs from gene duplications can confound many phylogenetic analyses, we employed a tree-based pruning approach meant to reduce misidentified orthologs and assembly errors (Yang & Smith 2014). The parameters used in this trimming procedure were derived based *a priori* knowledge of the Lecythidaceae phylogeny and inspection of hundreds of amino acid phylogenies (Methods S1; Mori *et al.*, 2015; Rose *et al.*, 2018; Larson *et al.*, 2020). The procedure included multiple sequence alignment with MAFFT v7.271 (Katoh *et al.*, 2002; Katoh & Standley, 2013) followed by amino acid tree estimation with RAxML v8.2.11 (Stamatakis, 2014) and was meant to reduce non-orthologous sequences in the orthogroup alignments, while minimizing loss of phylogenetic information for taxa in the Parvifolia clade (Methods S1). We use the term orthogroup to denote groups of sequences that appear to be reciprocally orthologous based on sequence similarity, regardless of their present function in individual species.

***Preliminary phylogenetic investigation*—**In order to identify clades of closely related individuals, check determinations for specimens, and to verify which individuals were nested within the Parvifolia clade, a phylogenetic tree (herein referred to as the preliminary phylogeny) was estimated with the assembled sequences from all 240 samples after the paralog filtering procedure described above. The preliminary phylogeny was estimated using RAxML v8.2.11 and a separate GTRCAT model partition for each of the exon and intron alignments of each orthogroup (Stamatakis, 2014). To assess support for clades in the preliminary phylogeny, rapid bootstrapping with 200 replicates was conducted. The results were visualized with Figtree (https://github.com/rambaut/figtree/).

***Genotyping and SNP analysis*—**In order to investigate the genetic structure of Parvifolia species and identify potentially admixed individuals, we called SNPs for each individual using GATK v.4.1.0.0 (McKenna *et al.*, 2010). The exon sequences for one individual for which we recovered 343 target loci

with a combined length of 836,403 bp were used as a reference assembly (Methods S2). Genomic variants were called for each individual following GATK best practices, with modifications where necessary to accommodate the available genomic resources for these non-model species (Methods S2; DePristo *et al.*, 2011; Li *et al.*, 2009; Li, 2013; Van der Auwera *et al.*, 2013; Poplin *et al.*, 2017; Hanlon *et al.*, 2019). Several clades were identified based on the preliminary phylogeny and a clade-specific SNP dataset in approximate linkage equilibrium was generated for each (Methods S2; Table S2; Purcell *et al.*, 2007). We used *Structure* v2.3.4 (Pritchard *et al.*, 2000) to investigate genetic clustering of individuals within each clade and determined the most appropriate number of populations (K) for each subset of taxa by comparing the estimated posterior probability of the data for multiple values of K in conjunction with *a priori* taxonomic information (Methods S2; Table S2). In cases where an individual showed strong evidence of clustering with a species other than that to which it was identified based on morphology, the identity of the individual was further investigated, and its determination was updated to reflect taxonomic uncertainty and all available evidence (Fig. S1; Methods S3). Special consideration was given to *E. roseocalyx*, which appeared to be nested within the broadly distributed species *E. coriacea* based on preliminary results (Methods S3; Batista *et al.*, 2017). To further explore patterns of genetic variation within *E. coriacea*, we used the gdsfmt and SNPRelate packages (Zheng *et al.*, 2012) in R v3.6.0 (R Core Team, 2019) to produce an additional SNP dataset and conducted a genetic principal component analysis (PCA), which was visualized with a custom R script that utilized the *plotly.js* library (Sievert, 2020).

***Verifying admixture with rooted triple tests*—**To corroborate the admixed ancestry of individuals identified using *Structure* and test for evidence of ancestral introgression among closely related species, we implemented a test capable of inferring admixture from a set of gene trees using rooted triplets (RT; i.e. gene trees consisting of three ingroup individuals and an outgroup; Fig. 2), which we conducted using the novel script *Run_RT_tests.py* (see Data Availability Statement). A version of this test has been proposed previously (Hudson *et al.*, 2005), but we are not aware of any previous studies that have used it to investigate admixture in target capture datasets. The RT tests were conducted by subsetting each orthogroup alignment to include the four individuals of interest and estimating a gene tree with branch lengths for each sub-alignment using IQ-TREE v1.6.3 (Nguyen *et al.*, 2014; Chernomor *et al.*, 2016). This obviated the need to re-align sequences for each test and allowed the sequence data from all 240 samples to inform the sub-alignment, which may have helped alleviate alignment issues due to missing data. Then, the topologies of the resulting trees were summarized to assess whether the data were compatible with a scenario of no admixture, using the same theoretical framework as the *D*-statistic (Green *et al.*, 2010). However, unlike most implementations of the *D*-statistic that count patterns in multiple sequence alignments or SNP datasets, our test is based on gene trees and can therefore readily be

used with phylogenomic datasets consisting of relatively large gene regions in which all sites within a region are assumed to share the same phylogenetic history.

When all four-taxon gene trees are rooted on a known outgroup, the result is a set of rooted triplets, each of which contains exactly one ingroup relationship. There is a single tree bipartition that contains topological information for the ingroup, since two individuals will be sister to the exclusion of the third. For a rooted triplet consisting of ingroup taxa A, B, and C, the three possible ingroup bipartitions are (AB|C), (AC|B), and (BC|A). We define the most frequent of the three bipartitions as the "major relationship" and the other two possibilities as "conflicting relationships". The two individuals that form the major relationship are inferred to be the two that are most closely related and are herein referred to as T_1 and T_2 (Fig. 2). T_1 and T_2 are assumed to share a most recent common ancestor (MRCA) that occurred more recently than the MRCA of all three ingroup individuals, whether or not there is ongoing gene flow between/within the population(s) to which T_1 and T_2 belong (i.e. they can be the same or different species). As long as there is a null expectation of no gene flow with the populations to which the third ingroup (T_3) or the outgroup (O) individuals belong (i.e. T_3 and O are different species from one another as well as from T_1 and T_2) and it can be assumed that for each gene tree, O has the earliest diverging sequence, then in the absence of gene flow between the lineages represented by T_3 and T_1 and/or T_2, the number of gene tree with each of the possible two conflicting relationships should be statistically equal, because each is equally likely to occur due to incomplete lineage sorting (ILS; Bryant & Hahn, 2020).

Any statistically significant deviation from equality can be considered evidence that the assumptions of the multispecies coalescent model have been violated by gene flow between the lineages to which T_3 and T_1 and/or T_2 belong. We calculate *P* as the probability of a result at least as unequal as the observed frequencies using a binomial test where each gene tree that conflicts with the major relationship represents a trial and the probability of either conflicting relationship is equal to 0.5.

To correct for multiple comparisons, we used the Holm-Bonferroni method with α=0.01 to adjust our critical value for rejecting the null hypothesis (Holm, 1979; Eaton *et al.*, 2015). The statistical power of each RT test is affected by the number of gene trees that conflict with the major relationship, which is expected to vary based on the time since the MCRA of the relevant individuals. The type II error rate (i.e. failing to reject the null hypothesis of no admixture when in fact there has been admixture) of this type of RT test may be relatively high for many target capture datasets, due to the relatively low number of independent trials available compared to some other tests for admixture using RADseq or whole genome assemblies. Because of this, our results may represent a conservative estimate of admixture among our sampled species, especially for cases of historical introgression involving small proportions of the genome. However, our statically significant results provide strong evidence of admixture.

It should be noted that because we utilized coding sequences and the introns adjacent to them, each locus is subject to natural selection. However, it is unlikely that selection would generally lead to a systematic bias for one conflicting gene tree topology over the other for a large enough number of independent loci to significantly increase the type I error rate (i.e. rejecting the null hypothesis of no admixture, when in fact no admixture has occurred). It is also important to note that the test as implemented does not explicitly account for heterozygosity, since each locus is represented by a single consensus sequence per sample, as is typical in most phylogenomic datasets. The effect that differing consensus-calling approaches during sequence assembly might have on phylogeny-based inferences of admixture warrants future study.

***Parvifolia clade phylogeny*—**To build a robust phylogenetic hypothesis for the Parvifolia clade, we conducted additional analyses without individuals with evidence of recent admixture. We used additional tree-based paralog pruning and generated two supermatrices, one that included data from introns and another that did not (Methods S4). For clarity, we refer to the best-scoring tree for the dataset that include both intron and exon sequences as the "Parvifolia phylogeny" and the best-scoring tree for the other supermatrix as the "exon-only Parvifolia phylogeny". For visualization, a version of each phylogeny was produced by trimming tips to include a single representative of each accepted species (Table S1) using the pxrmt function in phyx (Brown *et al.*, 2017). Conflict between the reduced-representation phylogenies was visualized using the phytools package in R (Revell, 2012). A version of the Parvifolia phylogeny with all tips, as well as an analysis of topological conflict with the untrimmed exon-only Parvifolia phylogeny, generated using the pxbp function in phyx, is also reported.

***Summaries of collection records and phenology for selected species*—**In order to visualize the extent of known range overlap among hyperdominant species *E. coriacea*, *E. parviflora*, *E. truncata*, and *E. wachenheimii*, we used a dataset curated by Mori *et al*. (2017) comprising available species occurrence records for these taxa (Vargas & Dick, 2020). All records for each species were plotted with QGIS v3.16.3 (https://github.com/qgis). We used a river shapefile available from the World Bank (https://datacatalog.worldbank.org/dataset/major-rivers-world, CC-BY 4.0 license), the World Borders Dataset (http://thematicmapping.org, CC BY-SA 3.0 license), and the digital elevation model of Lehner & Grill (2013). We plotted individual occurrences, rather than range summaries, to more clearly show the available data and corresponding gaps in existing collection records. To investigate flowering times of *E. coriacea*, *E. parviflora*, and *E. wachenheimii*, we used the C.V. Starr Virtual Herbarium (http://sweetgum.nybg.org/science/vh/) to examine all collections from Amazonas, Brazil housed at NY. We identified specimens with flowers or flower buds at time of collection and verified the collection date

and determination for each based on the specimen label. The results were plotted as box plots and dot plots for each species in R using ggplot2 (Wickham, 2016) after removing duplicate collections made from the same tree on the same day.

Results

***Admixture among species of the Parvifolia clade*—**Our SNP-calling approach identified 148,310 polymorphic sites among 109 individuals in the Parvifolia clade. Both *Structure* analyses and RT tests support evidence of admixture among two species pairs in our sampling. Two individuals collected at Reserve 1501 were supported as having near equal ancestry of *E. coriacea* and *E. wachenheimii* (Fig. 3). These individuals were not recovered as sister to one another in the preliminary phylogeny (Fig. S2) and RT tests showed significant evidence of admixture for separate tests that included these individuals (Table 2; Table S3). Two additional individuals were supported as genetic intermediates between *E. wachenheimii* (ca. 70-75% ancestry) and *E. parviflora* (ca. 25-30% ancestry) in *Structure*, with RT tests also supporting evidence of admixture (Fig. 3; Table 2; Table S3). This second pair of individuals were recovered as sister to one another in the preliminary phylogeny (Fig. S2).

We also tested for evidence of more ancient introgression among lineages using RT tests with three ingroup individuals from three different species determinations or *Structure* clusters (in cases where the individual's identity was unclear). Individuals whose determination contained an *affinis* modifier were considered to be their own lineage for this purpose. We conducted 25 such tests, selecting one individual per lineage and excluding individuals with evidence of recent admixture in *Structure* analyses. We did not find significant evidence of admixture in any of these tests (Fig. 4; Fig. S3; Table 2; Table S3), though three resulted in an uncorrected $P<0.05$ but that was not significant at the level of $\alpha=0.01$ after correcting for multiple tests with the Holm-Bonferroni method (Table 2; Table S3). One such test included individuals determined as *E. parviflora*, *E.* aff. *parviflora*, and *E. wachenheimii* in which 63.3% of conflicting gene trees supported one alternative ($P = 3.52\times10^{-3}$). Another test included individuals of *E. laevicarpa*, *E. bracteosa* and an individual determined as *E.* aff. *laevicarpa*: for this test 59.4% of conflicting gene trees supporting one alternative ($P = 8.58\times10^{-3}$). The third test including individuals of *E. truncata*, *E. coriacea*, and *E. sagotiana* resulted in 58.8% of conflicting gene trees supporting one alternative ($P = 1.19\times10^{-2}$; Table 2; Table S3).

***Monophyly of described species in the Parvifolia clade*—**In *Structure* analyses, individuals collected at Reserve 1501 were consistently assigned ancestry corresponding almost exclusively (i.e. greater than 95%) to a single cluster, with notable expectations for two individuals with evidence of admixture (Fig. 3-4; Fig. S4). There did not appear to be admixture within several clades based on samples collected at

Reserve 1501 including 1) *E. collina*, *E. bracteosa*, and *E. laevicarpa*, 2) *E. atropetiolata* and *E. cyathiformis*, or 3) *E. micrantha* and *E. rankiniae* (Fig. 4). When considering individuals from these species collected outside our focal plot, some were inferred to have ancestry corresponding to multiple species. However, this appeared to be the result of intraspecific variation due to geographic structure, as there was no evidence of admixture in relevant RT tests (Table S3). Intraspecific variation could have caused ancestry to be assigned to a second cluster due to the parameterization of the analysis or uneven sampling across subpopulations (e.g., several individuals sampled from Reserve 1501, one individual from another locality). Indeed, the tendency for *Structure* to assign mixed ancestry in the presence of isolation by distance (Pritchard *et al*., 2010) or when sampling is uneven across hierarchical levels of population structure (Puechmaille, 2016) has been well-documented. Alternatively, this signal could represent admixture that RT tests failed to detect.

Overall, most individuals had morphological determinations that agreed with genetic evidence. There were 60 individuals collected at Reserve 1501 with morphological determinations as one of our focal species or suspected hybrids. Of these, seven (11.7%) were shown to require redeterminations based on genetic evidence and two were shown to be admixed (Table S1). There were 51 individuals in our broader sampling of the Parvifolia clade that did not meet both of the following criteria: 1) determined to be a focal species based on morphology; and 2) collected at Reserve 1501. Of these 51, there were 11 (21.6%) that required redeterminations, and two that showed evidence of admixture. Seven could be redetermined to species and four were assigned a putative species determination with an *affinis* modifier to reflect uncertainty (Fig. S1; Table S1).

***Geographic structure in* E. coriacea—** There was strong evidence of geographic structure among 12 samples of *E. coriacea* with no evidence of recent admixture. In a PCA of SNP data, the first, second, and third principal components explained 15.14%, 11.24%, and 10.82% of the total variance respectively and individuals with the same country of origin clustered together (Fig. S5). In phylogenetic analyses, collections from Brazil formed a clade which was strongly supported as sister to collections from French Guiana (Table S1; Fig. S6). The single individual collected in Panama was sister to an individual collected at Los Amigos field station at Madre de Dios, Peru, with those sister to a clade of two individuals collected at Yasuní National Park in Ecuador; those four individuals were also inferred to have varying amounts of ancestry corresponding to a second cluster in *Structure* analyses, while individuals from Brazil and French Guiana had inferred ancestry almost exclusively corresponding to a single cluster (Fig. 3; Figs. S4).

***Phylogenetic relationships in the Parvifolia clade*—**Our target capture approach resolved most of the phylogenetic relationships among sampled species of the Parvifolia clade, though for some, support was dataset-dependent (Fig. 4; Fig. S6-7; Table S4). Seven relationships among accepted species differed between the Parvifolia phylogeny (i.e. intron and exon data) and the exon-only Parvifolia phylogeny (Fig. S7). Inferred relationships among individuals within a species tended to vary more than relationships among species across datasets (Fig. S6). Regardless of whether intron data was included, *E. truncata* and *E. wachenheimii* were inferred to be sister taxa, as were *E. coriacea* and *E. parviflora*. Those four species formed a clade with *E. sagotiana*, with that clade of five species sister to a clade consisting of *E. pedicellata*, *E. ovata*, and *E. albiflora* (Fig. 4).

***Summary of collection records and phenology of selected species*—**Existing collection records showed broad overlap in the geographic ranges of the four species we investigated (Fig. 5). Our survey of phenology yielded 63 unique collections in flower from Amazonas, Brazil (Table S6, see later). Collection date ranges and interquartile ranges for each of the three species overlapped, with the medians for each falling within three weeks of one another during the dry season (Fig. S8).

Discussion

***Admixture in the Parvifolia clade*—**Our results add to the small but growing body of evidence regarding admixture among tropical trees and are, to our knowledge, the first examples of nuclear admixture among hyperdominant Amazonian species. Our sampling included all accepted species of the Parvifolia clade known to occur in the intensively studied Reserve 1501 plot (Table S1; Mori & Lepsch-Cunha, 1995). All individuals of our 12 focal species collected at Reserve 1501 could be assigned robust species determinations based on *Structure* analyses and tree-based phylogenomic inference (Figs. 3-4; Table S1; Figs. S2-S4). Our results provide robust evidence of admixture between two of our focal species, *E. coriacea* and *E. wachenheimii*. The two *E. coriacea* × *wachenheimii* individuals were recovered as successively sister to all *E. wachenheimii* individuals in our preliminary phylogeny, consistent with each sharing a high degree of genetic similarity with *E. wachenheimii* while also harboring genetic dissimilarities with *E. wachenheimii* and with one another (Fig. S2). In addition, there was significant evidence for rejecting the null hypothesis of no admixture for RT tests that included one *E. wachenheimii*, one *E. coriacea*, and either putative *E. coriacea* × *wachenheimii* individual (Table 2; Table S3).

Our results also strongly support hypotheses of admixture between *E. wachenheimii* and *E. parviflora* (Fig. 3; Table 2). The *E. parviflora* × *wachenheimii* individuals were inferred to have unequal ancestry from the two parent species, suggesting that hybridization followed by backcrossing may have occurred (Fig. 3; Fig. S4). We note that only a single individual of *E. parviflora* has ever been recorded at

Reserve 1501 and therefore was not among our focal species; the collections of these admixed individuals were made within the BR-319 plot network, south of Reserve 1501 (https://ppbio.inpa.gov.br/sitios/br319; Table S1). Both sampled individuals with >95% ancestry corresponding to *E. parviflora* in *Structure* analyses were collected in French Guiana. However, our results are not consistent with geographic structure: the relevant RT tests rejected the null hypothesis of no admixture for triplets consisting of one *E. wachenheimii*, one *E. parviflora*, and either putative *E. parviflora* × *wachenheimii* intermediate (Table 2; Table S3).

All three of species with evidence of admixture, *E. coriacea*, *E. wachenheimii*, and *E. parviflora*, have been described as hyperdominant—members of a group of 217 tree species that comprise approximately 50% of the tree numbers and biomass of Amazon forests (ter Steege *et al*., 2013). *Eschweilera coriacea* is the third most abundant tree species across an Amazon-wide network of forest inventory plots, with an estimated census population size of between four and five billion individuals (ter Steege *et al.*, 2013, 2020) and is the only tree species to be considered hyperdominant in both the Amazon basin and Guiana Shield regions (ter Steege *et al*., 2013).

Is admixture widespread among species of* Eschweilera*?— Given the sizable gaps in available data on hyperdominant species of *Eschweilera*, additional research is clearly needed to reveal the full extent of admixture among them. We found admixture between two species pairs of hyperdominant *Eschweilera* at two different localities, despite sampling 12 or fewer individuals for any species (Table 1). Of the three individuals suspected to be hybrids based on morphology, only one showed evidence of admixture, while three other individuals, one originally determined as *E. coriacea* and two as *E. truncata*, were also found to be admixed (Table S1). This suggests that trees with or without obvious morphological signs of hybridity may have admixed genomes. Data on the phenology of these species is quite limited but indicates that broad overlap in flowering times during the dry season cannot be ruled out based on existing data (Fig. S8) and evidence of admixture clearly demonstrates that phenological overlap can occur in the Amazon basin.

Given the current evidence, the large population sizes of these species, their large and frequently overlapping ranges (Fig. 5; Mori *et al.*, 2017), and the prevalence of gene tree conflict in our results (Table S3), we argue that admixture among *E. coriacea*, *E. wachenheimii*, and *E. parviflora* may be extensive and that future efforts are likely reveal further evidence that admixture has played a role in the evolution of these and possibly other species of *Eschweilera*. However, deeper sampling is necessary to determine the extent of admixture and whether additional species admix. The results of several RT tests showed patterns of gene tree conflict suggestive of ancestral evolutionary reticulations, but that failed to meet our criteria for statistical significance (Table 2). Future work that implements explicit tests for

admixture with more independent loci may provide stronger evidence regarding whether ancient evolutionary reticulations have occurred in *Eschweilera*. Future sampling efforts with a larger geographic focus could also produce quantitative estimates of gene flow among lineages across the Neotropics, and investigate whether entire populations, rather than individuals, bear genomic signatures of admixture.

Biological implications of admixture among dominant tropical lineages—If admixture is widespread, interspecific gene flow may be an important factor in the evolution of the Parvifolia clade and could shape their reproductive biology, local adaptation, and ecological interactions. Hybridization and introgression can have various outcomes including increasing genetic diversity, sharing of adaptive alleles, and either increasing or decreasing the strength of reproductive isolation barriers (Rieseberg & Wendel, 1993). In some cases, a complete breakdown of reproductive isolation barriers can cause "lineage collapse" or "speciation reversal", resulting in a new lineage with a mosaic genome (Kearns *et al*., 2018). Alternatively, if hybrids are inviable, prezygotic isolation barriers may evolve (i.e. reinforcement) or there may be little or no lasting population level effects of hybridization. In the case of *Eschweilera*, current evidence suggests that chloroplast capture may be quite common (Huang *et al*., 2015; O. Vargas *et al*., unpublished), indicating that at least some hybrids are capable of backcrossing with their parent species.

Our results show that morphologically defined species largely correspond to distinctive gene pools in our focal species, even in those that admix. The continued genetic cohesion of admixing species could be due to several factors including hybrid inviability or divergent selection acting on suites of traits that differ among these species. Unfortunately, data about the reproductive biology and ecology of the species found to admix are limited. All three species most often occur in non-flooded forests, though *E. coriacea* appears to tolerate flooding more readily than the other two (Mori & Lepsch-Cunha, 1995; Mori *et al.*, 2010 onward). *Eschweilera coriacea* frequently reach the canopy while *E. wachenheimii* are typically smaller and occupy the understory. *Eschweilera parviflora* are most often found in the understory, but can also reach the canopy (Mori *et al.*, 2010 onward). All three species differ somewhat in floral morphology (Fig. 1A-C; Table S5) and may attract different pollinators, though observations of floral visitors are lacking for these species (Mori *et al.*, 2010 onward). A better understanding of the nuanced ecological differences among these species may help shed light the selective forces that maintain their genetic separation.

A group of taxa that remain largely distinct despite incomplete reproductive barriers is sometimes called a syngameon (Lotsy, 1925; Suarez-Gonzalez *et al.*, 2018). Several of the best-studied examples of syngameons in trees are found within the oaks (*Quercus*), which hybridize prodigiously (e.g. Eaton *et al.*, 2015; Hipp *et al.*, 2020), yet largely retain their cohesion as species (Hardin, 1975; Cavender-Bares,

2019; Kremer & Hipp, 2020) and likely facilitate one another's ecological success through introgression (Leroy *et al.*, 2020). Our results suggest that some members of the Parviflora clade including *E. coriacea*, *E. wachenheimii*, and *E. parviflora* could represent a syngameon, which have been hypothesized to be common in tropical trees (Cannon & Lerdau, 2015; Schmitt *et al*., 2021), but have not often been documented with genomic evidence. Exchanging genes with other species might facilitate local adaptation across the broad ranges of species like *E. coriacea* (Fig. 5), but further investigation is needed to test for evidence of a relationship between admixture, species abundances, and ecological amplitude.

***Population structure*—**We found evidence of geographic structure within the hyperdominant species *E. coriacea* (Fig. 3; Figs S4-5). In *Structure* analyses, runs with the best posterior probability consistently inferred individuals of *E. coriacea* to correspond to two clusters, with individuals assigned varying proportions of the two clusters depending on where the specimen was collected, in a gradient from Panama to Ecuador and Peru to French Guiana and Brazil (Fig. 3; Fig. S4). We also found evidence to suggest population structure in other species in the Parvifolia clade, including *E. truncata* (Fig. 3; Fig. S4), *E. sagotiana* (Fig. S4), *E. collina* (Fig. 4), and *E. pedicellata* (Fig. 4), though we note our sampling was not designed to make inferences on geographic structure in these species. Phylogeographic structure is expected within broadly distributed Neotropical trees (Dick & Pennington, 2019) and has previously been uncovered in several species (e.g. Dick & Heuertz, 2008; Nazareno *et al*., 2019).

***Implications for the taxonomy of Amazonian trees*—**While a reassessment of species limits is outside the scope of this work, our results suggest that our focal species can be robustly identified with the methods we employed (Figs. 2-3; Figs. S3-4). Despite the occurrence of admixture in some species, most individuals identified as a focal species clustered with other individuals with the same morphological species identification (Table S1). Our results therefore suggest that admixture has not led to the widespread erosion of species boundaries within the clade and therefore, morphology can be used to reliably distinguish among most co-occurring species of Lecythidaceae. However, cases in which genomic evidence did not match existing determinations suggest that refined taxonomic and genetic studies may be warranted for some species including *E. coriacea* and *E. micrantha* (Table S1).

Our results show that morphological determinations for specimens collected outside Reserve 1501 more frequently conflicted with genomic evidence than did determinations for specimens from the intensively studied plot (Table S1). Intraspecific morphological variability, identification errors, as well as admixture may have contributed to this discordance. Many species in the Parvifolia clade, including the three species for which we find evidence of admixture, have similar vegetative characteristics (Table S5), overlapping phenology (Fig. S8; Table S6), and are broadly distributed across the Neotropics (Fig. 5;

Mori *et al.*, 2010 onward; Mori *et al*., 2017). Our results suggest that the methodology employed here might be useful for investigating species delimitation in relation to the geography of broadly distributed tropical tree species. To better characterize species boundaries in tropical trees, studies should explicitly investigate morphological characters in conjunction with genomic evidence, including for admixed individuals and/or populations.

***Utility of target capture for studying tropical tree populations*—**There are several methods available to detect evidence of admixture, each with its own benefits and assumptions. The target capture protocol employed here, with probes specifically designed to recover low copy number, genetically variable loci in Lecythidaceae (Vargas *et al.*, 2019), allowed us to investigate evolutionary history using phylogenetic and Bayesian clustering approaches. Our inferences were based on highly variable coding regions, which may be under natural selection. The effect that targeting such regions has on studies of admixture and species delimitation warrants further study. While employing neutral markers or a larger number of loci may have led to different estimates of ancestry, our dataset allowed us to identify admixed individuals and distinguish intraspecific geographic variation from admixture using an explicit test. There are drawbacks to using target capture at infraspecific phylogenetic scales, including the relatively high per sample cost compared to other reduced-representation genome sequencing approaches such as RADseq. However, RADseq protocols often require relatively high-molecular weight input DNA (Graham *et al*., 2015), while target capture can more readily allow researchers to include samples from partially degraded herbarium specimens (Brewer *et al.*, 2019). We recovered sequences from 343 loci, far fewer than often recovered with RADseq, but far more than most studies that use microsatellites. However, unlike for many RADseq datasets, we recovered sequence data for nearly all target loci for most samples (average 339.6 of 344 loci/individual), which enabled gene-tree based methods for explicitly testing hypotheses of admixture. Our results suggest that target capture can be used to study admixture in topical trees and may be especially useful for studies that wish to include herbarium specimens.

Author Contributions

D.A.L. and C.W.D. conceived the study. A.V. hosted the field work, obtained collection and export permits, and provided access to existing BDFFP collections. D.A.L. and C.W.D. conducted the field work to obtain new collections. D.A.L. conducted the lab work for focal species and designed and performed the analyses. O.M.V. led the sampling and lab work for the broader phylogeny. O.M.V. and D.A.L. mapped collection records. The figures and tables were prepared by D.A.L. The manuscript was written by D.A.L. with editing by C.W.D. and input from all authors. All authors contributed to and approved the final version of the manuscript.

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Data Availability Statement

The data, novel scripts, and output files that support the findings of this study are openly available from Dryad at doi:10.5061/dryad.fj6q573t4. Raw sequence reads are available from NCBI BioProject PRJNA641333.

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Fig. S1 Schematic of the process for making redeterminations based on all available evidence.

Fig. S2 The preliminary phylogeny of the Parvifolia clade, estimated from a supermatrix of intron and exon target capture data.

Fig. S3 Results of several *Structure* analyses with alternative values of K.

Fig. S4 Results of *Structure* analyses using a SNP dataset for the clade that included *E. coriacea*, *E. wachenheimii*, *E. sagotiana*, *E. truncata*, and *E. parviflora*.

Fig. S5 Three-dimensional scatterplot for a genetic principal component analysis showing geographical

structuring in samples of *E. coriacea*.

Fig. S6 The Parvifolia phylogeny without reduced representation, produced using a supermatrix of intron and exon target capture data.

Fig. S7 Comparison of the reduced-representation Parvifolia phylogenies recovered with two supermatrices.

Fig. S8 Boxplots overlayed with dot plots showing the day of the year that collections were made for specimens at the New York Botanical Garden Herbarium.

Table S1 Voucher and accession information for samples used in the study.

Table S2 Summary statistics for all SNP datasets and estimated probability of the data for all *Structure* analyses for differing values of K.

Table S3 Summary of all rooted triplet tests conducted.

Table S4 Results of tree searches and likelihood recalculations for the Parvifolia phylogenies, ordered by increasing AIC score.

Table S5 Summaries of morphological and ecological traits of species inferred to engage in admixture.

Table S6 Information for flowering specimens collected in Amazonas, Brazil and housed at the New York Botanical Garden Herbarium.

Methods S1 Paralog filtering and alignment.

Methods S2 Genotyping and SNP dataset analyses.

Methods S3 Redetermination of individuals based on all available evidence.

Methods S4 Parvifolia phylogeny supermatrix construction and phylogeny estimation.

Notes S1 Note on species of the Parvifolia clade at Reserve 1501.

Notes S2 Notes on sampling at Reserve 1501 and prioritization of morphological intermediates.

Group or focal species	Number of samples (based on morphology in parentheses)	Named spp. represented
E. atropetiolata S.A.Mori	5 (5)	1
E. bracteosa (Poepp. ex O.Berg) Miers	4 (6)	1
E. collina Eyma	5 (5)	1
E. coriacea (DC.) S.A.Mori	12 (13)	1
E. cyathiformis S.A.Mori	5 (4)	1
E. laevicarpa S.A.Mori	7 (6)	1

E. micrantha (O.Berg) Miers	2 (6)	1
E. pedicellata (Rich.) S.A.Mori	6 (7)	1
E. pseudodecolorans S.A.Mori	5 (4)	1
E. rankiniae S.A.Mori	4 (4)	1
E. truncata A.C.Sm.	10 (9)	1
E. wachenheimii (Benoist) Sandwith	4 (6)	1
Focal species or admixed from Reserve 1501	58 (60)	12
Admixed within Parvifolia clade	4 (3)	n.a.
Parvifolia clade	109 (107)	33
Lecythidaceae	240 (240)	127

Table 1. Summary of the number of samples before and after making redeterminations.

Samples forming major relationship	**Third ingroup**	**Major relationship count**	**Conflict 1 count**	**Conflict 2 count**	***P* value**	**Corrected crit. value**	**Reject H-null**
EswaL779, EscoL796	EscoL834	141	124	53	4.87E-08	2.22E-04	Yes
EstrL882, EswaL779	EspaL068	134	124	56	2.18E-07	2.27E-04	Yes
EswaL779, EscoL824	EscoL834	129	126	59	4.69E-07	2.33E-04	Yes
EstrL891, EspaL068	EswaL779	131	113	64	1.42E-04	2.38E-04	Yes
EsmiL332, EspaL068	EswaL779	208	69	40	3.52E-03	2.44E-04	No
EscoL885, EslaL783	EsbrL794	147	101	69	8.58E-03	2.50E-04	No
EstrL838, EscoL834	EssaL335	140	104	73	0.012	2.56E-04	No
EsroL664, EsamL886	EsmiL823	120	111	83	0.026	2.63E-04	No
EscoL241, EscoL828	EscoL885	224	50	36	0.080	2.70E-04	No
EscoL771, EswaL839	EstrL711	212	55	42	0.111	2.78E-04	No
EsteL690, EstrL772	EspaL068	222	50	38	0.120	2.86E-04	No
EstrL838, EswaL779	EssaL335	170	80	66	0.141	2.94E-04	No
EspaL386, EspaL868	EsteL704	221	49	39	0.169	3.03E-04	No
EscyL797, EsrhL578	EsatL643	164	77	65	0.178	3.13E-04	No
EstrL838, EswaL779	EscoL834	118	107	93	0.179	3.23E-04	No

Table 2. Summary of 15 rooted triplet tests, ranked in order of increasing *P* value.

Figure legends

Figure 1. Examples of the morphology of members of the Parvifolia clade. A) Flower of *Eschweilera parviflora*. B) Lateral view of a flower of *Eschweilera wachenheimii*. C) Flower of *Eschweilera coriacea*. D) Flower of *Eschweilera collina* with androecial hood sectioned. E) Fruit bases, opercula, and seeds of *Eschweilera parviflora*. F) Fruits, operculum, and seeds of *Eschweilera coriacea*. G) Leaves and old fruit of *Eschweilera atropetiolata*. H) Abaxial view of a leaf of *Eschweilera coriacea*. I) Bark of *Eschweilera tessmannii*. J) Bark of *Eschweilera truncata*. K) Bark of *Eschweilera sagotiana*. L) Bark of *Eschweilera atropetiolata*. Photo attribution: A, B, E, F, G, I, J & L to Scott Alan Mori; C, D & K to Carol Ann Gracie; H to Xavier Cornejo. Reproduced under terms of the CC BY-NC-SA 3.0 license. Captions are adapted from Mori *et al*. (2010 onward).

Figure 2. Schematic of the rooted triplet test for assessing evidence of admixture. Red arrows indicate four hypothetical samples selected for the test. The test assumes that the outgroup diverges first in all gene trees and at least two species are represented in the ingroup. Blue and red phylogenies represent the two possible topologies that conflict with the most common topology after all possible gene trees have been generated. Any statistically significant deviation from equal numbers of the two conflicting topologies, where *P* is the probability of a result at least as unequal as the observed frequencies using a binomial test, is considered evidence that the assumptions of the multispecies coalescent have been violated by admixture among species.

Figure 3. Population structure (K=5) of all samples in the clade that included *E. coriacea*, *E. wachenheimii*, *E. truncata*, and *E. parviflora*. Each bar represents the ancestry of an individual inferred with *Structure*. Each individual is labeled with a unique code used throughout all analyses and asterisks indicate samples from focal species collected at Reserve 1501. Collection locations outside Reserve 1501 are indicated as follows: Pa-Panama, Pe-Peru, E-Ecuador, F-French Guiana, B-Brazil. Black stars above bars indicate individuals with significant evidence of admixture based on an RT test.

Figure 4. Phylogeny of the Parvifolia clade visualized using a single representative per accepted species. Branch labels are IQ-TREE ultrafast bootstrap support. Asterisks on branch labels indicate nodes that conflict with the best scoring maximum likelihood topology recovered with an exon-only supermatrix. The results of *Structure* analyses are shown for SNP datasets that included all individuals within the corresponding clades indicated on the phylogeny. Each individual is labeled with a unique code used

throughout all analyses and asterisks on these labels indicate samples from focal species collected at Reserve 1501. The legend for each sub-plot indicates the one or more species that most closely corresponded to each cluster based on accepted taxonomy. The individuals in these clades generally clustered along morphologically defined species boundaries and there was no significant evidence of admixture for these taxa based on rooted triplet tests.

Figure 5. Occurrence records across the Neotropics for four closely related species in the Parvifolia clade, three of which show evidence of admixture in this study. Few or no records are available for these species across much of the Amazon basin since most come from collections made in permanent plots. Because of this, there is uncertainty in the true extent of range overlap among these and other species of Lecythidaceae as well as many other clades of Neotropical trees.

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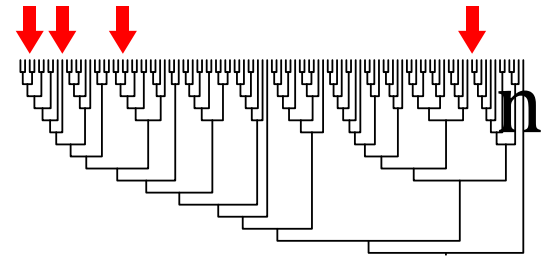
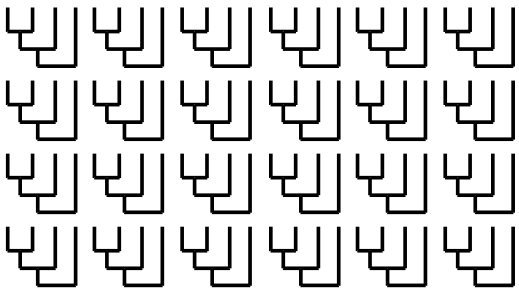
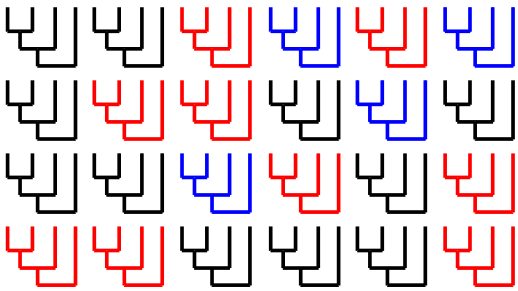


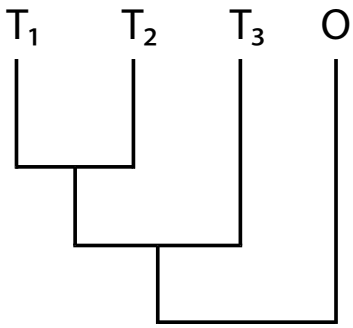
Select three individuals of two or more species among which to test for evidence of admixture as well as a more distantly related individual to use as an outgroup for rooting gene trees



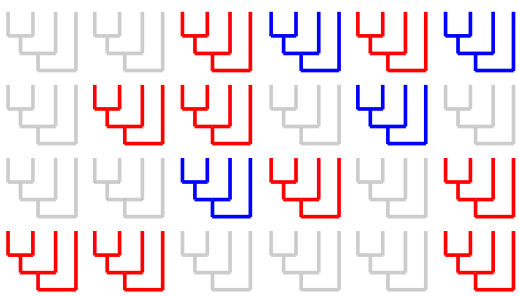
Generate all possible rooted gene trees



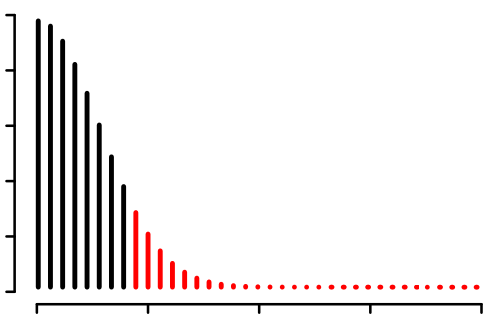
Summerize the results to find the most common gene tree topology (black) among the three topological possibilities



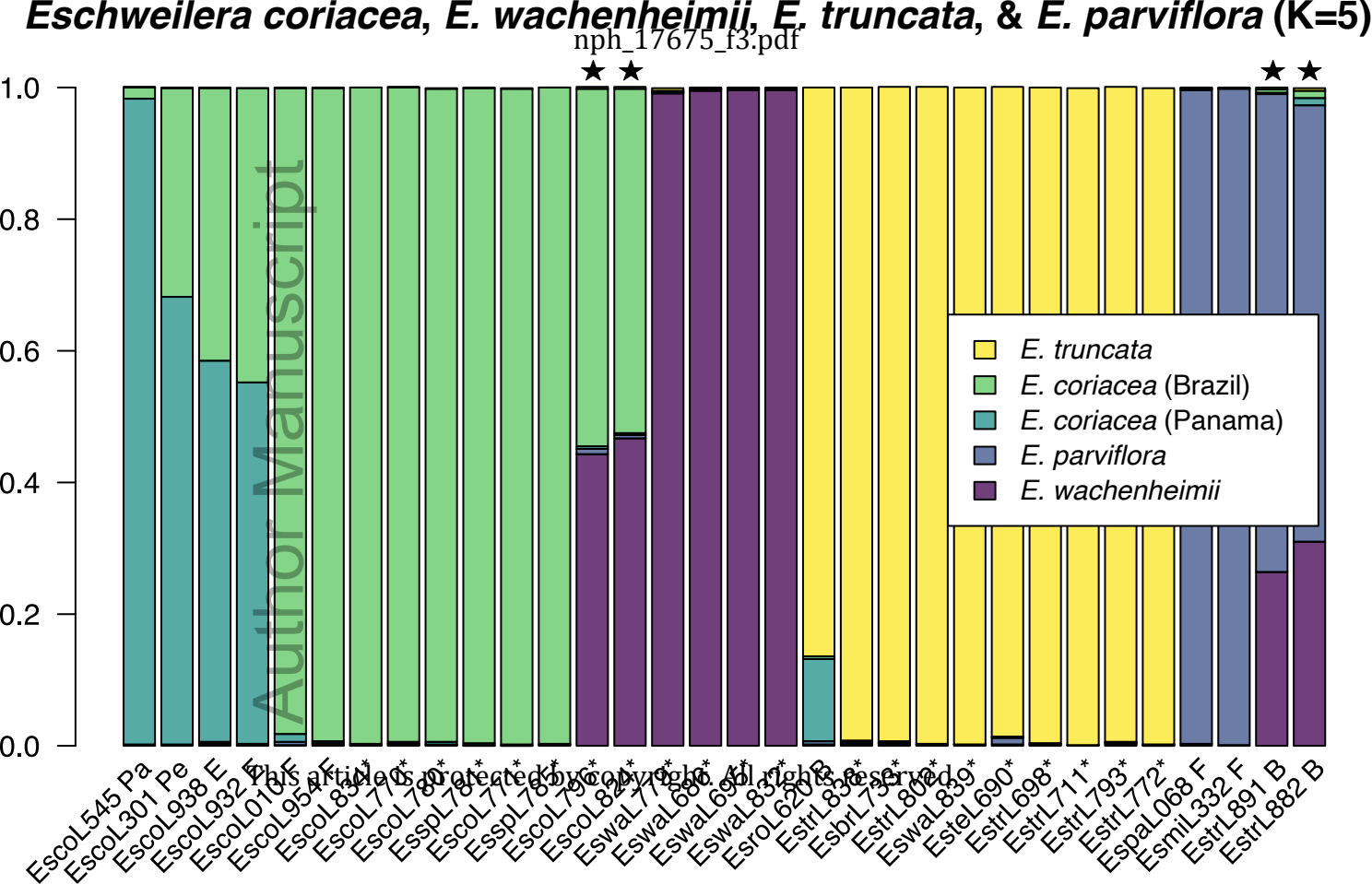
The most common topology is defined as the major relationship, with individuals T_1 and T_2 more closely related to one another than to T_3



Determine how many gene trees contain each of the two possible topologies that conflict with the major relationship



Calculate the probability (*P*) that the more common conflicting topology could occur at least as many times as actually observed if the null hypothesis of no admixture is true and the observed gene tree conflict is due to ILS



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