



# Diversification of the orchid genus *Tridactyle*: Origin of endemism on the oceanic islands of São Tomé & Príncipe in the Gulf of Guinea

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## Abstract

**Aim:** Oceanic islands have played an important role in our understanding of the diversification of organisms, and phylogenetic estimates have been used in this context to investigate the origin of island diversity and its relationship to the continent. Using a typical orchid genus rich in island endemics and with widespread continental relatives, we aim to compare alternative hypotheses of diversification with a focus on island endemism.

**Location:** Tropical Africa and the Gulf of Guinea Islands of São Tomé & Príncipe, Central Africa.

**Taxon:** *Tridactyle* genus (Orchidaceae).

**Methods:** We used genome skimming to sequence the whole chloroplast genome and nuclear ribosomal genes from 157 individuals of 34 *Tridactyle* species and 15 individuals of 12 other orchid genera (outgroups) to infer a time-calibrated phylogenetic tree of the genus *Tridactyle*. We also used multiple statistical methods to infer the geographical ranges of the ancestral nodes from the estimated phylogeny. Alternative hypotheses for the origins of endemism on the islands of São Tomé and Príncipe were investigated based on the biogeographical reconstruction of the genus.

**Results:** The estimated phylogeny of *Tridactyle* and reconstruction of geographical ranges for the ancestral nodes suggested a general history of allopatric speciation for the genus, in particular via colonization of the islands of the Gulf of Guinea that induced a long period of geographical isolation. The most parsimonious hypothesis to explain island endemism in *Tridactyle* involved six independent colonizations of the islands from the continent.

**Main conclusions:** In contrast to other cases of oceanic island endemism that involved adaptive radiation on an island or within an archipelago, endemism in *Tridactyle* is better explained by multiple colonization events from the continent to São Tomé and Príncipe, with subsequent divergences due to geographical isolation but only one potential instance of further diversification on the islands.

## KEYWORDS

African rainforests, evolutionary history, high-throughput sequencing, island biogeography, molecular dating, Orchidaceae, phylogeography, plastome captures, *Tridactyle*

## 1 | INTRODUCTION

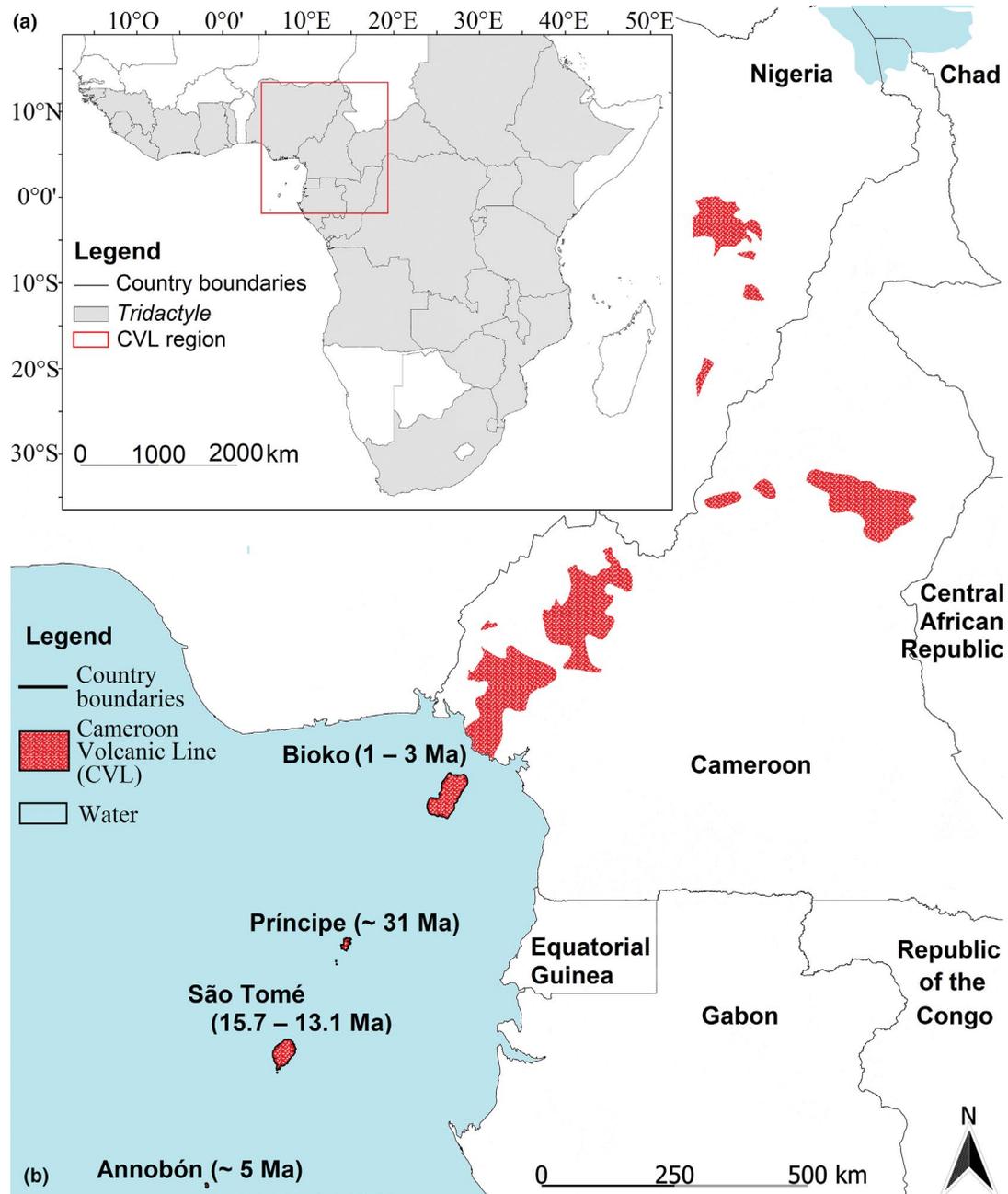
Oceanic islands have played an important role in investigating the processes governing biological diversity (Mayr, 1942; Wilson & MacArthur, 1967). In their seminal book, entitled *The Theory of Island Biology*, Wilson and MacArthur (1967) presented a formal model for predicting how the dynamics of colonizations and extinctions shaped island diversity. More recently, the possibility of inferring phylogenetic trees from DNA sequence variation allowed biologists to go beyond the equilibrium model of MacArthur and Wilson and to investigate the origin of current island diversity in an evolutionary context (Emerson, 2002). In particular, two alternative models of speciation have been proposed to explain island endemism and phylogenetic studies are now routinely used to discriminate between them. First, adaptive radiation on the island (or island archipelago) can occur following a single colonization from the continent. This model is illustrated by the famous Galapagos finches which helped Darwin conceptualize his theory of evolution. Inferring their phylogeny suggested a single colonization of the archipelago from South America followed by the diversification of this ancestor through adaptation to multiple environmental niches (Emerson, 2002; Sato et al., 1999, 2001). The exceptionally high diversity of Hawaiian endemic lobeliads (Campanulaceae) offers another example, with the phylogenetic study of Givnish et al. (2009) showing they form a monophyletic group and are thus likely the product of a single immigration event that occurred  $\pm 13$  Mya, followed by a process of adaptive radiation. In the second model, multiple colonizations to the island (or island archipelago) occur from the continent, without subsequent speciation on the island. This model was referred as 'anagenetic evolution' by Stuessy et al. (2006), who estimated that it concerned, on average, 25% of endemic plant species on oceanic islands. Note that Emerson and Patiño (2018) have convincingly argued that the term anagenetic evolution was not necessarily appropriate for all endemic island species that did not radiate afterwards, because it implies evolutionary stasis, while we usually have no information on the evolutionary changes that occurred within species.

Other phylogenetic studies have suggested a more complex history of exchanges between islands and continents, and have challenged the traditional view (e.g. Wilson, 1961) that oceanic islands are solely biodiversity sinks, and not a source for continental diversity (Bellemain & Ricklefs, 2008; Emerson, 2002). For example, Carine et al. (2004) suggested based on a molecular phylogeny of the genus *Convolvulus* (Convolvulaceae) two independent introductions in Macaronesia, and one subsequent back-colonization to the continent. Overall, a number of evolutionary studies involving both plants and animals provide indeed increasing evidence that speciation on islands can produce a large reservoir of diversity (Bramwell & Caujapé-Castells, 2011; Caujapé-Castells, 2004; Fernández-Mazuecos & Vargas, 2011; Hutsemekers et al., 2011; Nieto-Blázquez et al., 2017), which can later provide an important source of diversity for the continent via back-colonization (Carine et al., 2004; Caujapé-Castells, 2004; Filardi & Moyle, 2005; Patiño et al., 2015). For instance, Caribbean anoles, an iconic example of island radiation, have

been found to constitute an important source of lizard diversity for mainland South America (Nicholson et al., 2005). In this context, we present here a phylogenetic study comparing multiple species from the orchid genus *Tridactyle* found on the islands of São Tomé and Príncipe (Gulf of Guinea) and in continental Africa.

The Cameroon Volcanic Line (CVL) was identified as a strong phytogeographical boundary separating the bioregions of Upper and Lower Guinea (Droissart et al., 2018). Its southern end is composed of four islands: Bioko (formerly Fernando Po), São Tomé, Príncipe and Annobón (Figure 1). São Tomé and Príncipe are considered as a distinct bioregion by Droissart et al. (2018). Both islands are known to be an important centre of endemism (Droissart et al., 2018; Jones, 1994; Sosef et al., 2017), particularly for birds (17 single-island endemic species and 3 endemic species shared by São Tomé and Príncipe out of a total of 50 species, de Lima et al., 2017), but also for a 'poor-dispersing' fauna, such as amphibians (Bell, Drewes, Channing, et al., 2015; Bell & Irian, 2019; Gilbert & Bell, 2018; Measey et al., 2007), reptiles (Ceriaco et al., 2016, 2017, 2018; Dallimer et al., 2009; Jesus et al., 2007; Jesus et al., 2009) and shrews (Ceriaco et al., 2015; de Lima et al., 2016). The prevailing explanation for this high rate of endemism is the long distance separating them from the continent; their colonization probably occurred via punctual rafting events following the Congo, Niger and Volta Rivers (Ceriaco et al., 2017; Drewes & Wilkinson, 2004; Measey et al., 2007), or even transoceanic events (Daniels & Klaus, 2018). A single colonization during the Miocene to early Pliocene, from West-Central Africa and subsequent diversification within the Gulf of Guinea island chain is proposed for the frog genus *Hyperolius* (Bell, Drewes, Channing, et al., 2015) and for the green snakes *Philothamnus* (Jesus et al., 2009). For the frog genus *Phrynobatrachus*, however, multiple colonization events might have taken place, as each species present in the archipelago is more closely related to a mainland sister species (Zimkus et al., 2010). Multiple colonizations can also be inferred from the phylogenetic relationships retrieved for the *Mabuya* spp. skinks (Jesus, Harris, & Brehm, 2005), *Hemidactylus* geckos (Jesus, Brehm, & Harris, 2005) and freshwater crabs *Potamonautes* (Daniels & Klaus, 2018). Consequently, the two islands are characterized by different biogeographical faunal histories, the fauna of Príncipe showing more affinities with the Guineo-Congolian fauna (Upper Guinea, Lower Guinea and Congolia) (Ceriaco et al., 2015; Daniels & Klaus, 2018), while that of São Tomé shows more affinities with the Lower Guinea and East African fauna (Ceriaco et al., 2016; Daniels & Klaus, 2018; Jesus, Harris, & Brehm, 2005; Loader et al., 2007).

To date, no quantitative biogeographical study has been conducted on the flora of the archipelago. Plana et al. (2004) have inferred a dated phylogeny of the African *Begonia* L. species, although they did not specifically estimate the geographical range of the ancestral nodes. Their phylogeny nevertheless indicates that two endemic lineages from São Tomé are each sister to clades including species that are either exclusively from the mainland or found on both the mainland and São Tomé. The uncovered phylogenetic pattern for the endemic species could indicate two separate colonizations of the island and one speciation event on the island.



**FIGURE 1** (a) Distribution map of *Tridactyle* Schltr. (b) Major geological features of the Cameroon Volcanic Line (CVL) and age of the Gulf of Guinea islands. Country abbreviation: CAR (Central African Republic) (Adapted from OneGeology Portal, French Geological survey (BRGM), Thiéblemont D. (edit.) et al., 2016, Geological Map of Africa at 1:10 M scale, CGMW-BRGM 2016)

The authors suggest that four species found on both the island and the continent have recently colonized São Tomé from the mainland. Overall, the study suggests the genus has been able to colonize São Tomé multiple times from the continent.

The Orchidaceae, in particular, have been intensively inventoried and collected on the archipelago over the last 20 years, leading to several publications of new species and taxonomical inventories (Cribb et al., 1999; Droissart, 2009; Droissart et al., 2009; Ječmenica et al., 2017; Stévant et al., 2000; Stévant & Cribb, 2004a, 2004b; Stévant & Geerinck, 2000, 2001). The orchids and their minute seeds are usually considered as having good dispersal

capacities (Di Musciano et al., 2020; Micheneau et al., 2008), but appropriate mycorrhizal symbionts are required for full establishment (Dressler, 1981). The family now counts 21 endemic species out of 104 on São Tomé, corresponding to 20% of endemism, but only 5 endemic species out of 71 on Príncipe, corresponding to 7% of endemism (Droissart, 2009). In this study, we focused on the genus *Tridactyle* Schltr., a sub-Saharan genus of 47 species, that includes 6 species on São Tomé and Príncipe, of which 3 are endemics (Figueiredo et al., 2011). There are no *Tridactyle* on Annobón (Velayas et al., 2014), and the four species present on Bioko are widely distributed on the continent, except for *T. exellii* that is endemic to the

CVL (Velayos et al., 2013). We first inferred a phylogenetic estimate of the genus based on DNA sequences of the complete chloroplast genome and of the nuclear ribosomal genes. We then used the estimated tree for conducting a biogeographical analysis, to characterize the mechanisms of diversification of this genus in the Gulf of Guinea Islands (GGI). In particular, our goal was to explore the movements that occurred during the evolution of the genus between the islands and the continent.

More specifically, we asked three separate questions. First, is the presence of *Tridactyle* species on the islands the result of a single colonization event from the continent followed by subsequent diversification on the islands, or did multiple colonization events occur from the mainland to the islands? Second, has further diversification of the genus occurred on the islands? Finally, can back-colonization events from the islands to the continent can be highlighted in *Tridactyle*?

## 2 | MATERIALS AND METHODS

### 2.1 | Taxon sampling

In all, 157 specimens from 34 species of *Tridactyle* (out of 47) were sampled across the African continent and São Tomé and Príncipe. The CVL southern end is composed of four islands: Bioko (formerly Fernando Po), São Tomé, Príncipe and Annobón (Figure 1). Bioko lies at about 35 km of Mount Cameroon (Yamgouet et al., 2015) and is the largest (2000 km<sup>2</sup>) island of the archipelago (Fa et al., 2000). It is a land-bridge island that has been connected to the continent multiple times since its emergence 1–3 Ma ago (Bell et al., 2017; Marzoli et al., 2000; Meyers et al., 1998). The smallest (17 km<sup>2</sup>), youngest (4.9 Ma) and farthest (335 km from the continent) of the oceanic islands is Annobón (Lee et al., 1994). The two others, São Tomé and Príncipe, are separated by 140 km of open water and form together the second smallest country of Africa after the Seychelles (1001 km<sup>2</sup>, combined). Príncipe is the oldest (31 Ma), smallest (128 km<sup>2</sup>), closest island to the continent (225 km off the West African coast) and reaches 948 m above sea level (Daniels & Klaus, 2018) while São Tomé is younger (15.7–13.1 Ma) (Lee et al., 1994), larger (836 km<sup>2</sup>), farther from the continent (240 km off the coast) and reaches 2024 m above sea level (Daniels & Klaus, 2018). Most of the samples were obtained from fieldwork and the shadehouse network from Central Africa (Stévant, 2003; Stévant et al., 2020), from São Tomé, Príncipe, Equatorial Guinea, Guinea Conakry, Cameroon, Gabon, Rwanda, Kenya, Tanzania and South Africa. Plants from Cameroon and Gabon were grown in shadehouses monitored by our teams in Yaoundé (for those collected in Cameroon) and in Libreville, Tchimbélé and Sibang (for those collected in Gabon). Herbarium specimens from the Kew Botanical Garden collection were sampled, as well as living plants from the Botanical Garden of Meise. The coverage of our sampling is extensive and covers the entire geographical distribution of *Tridactyle*, including East Africa, one of the centres of diversity of the genus, for which our sampling included

60% of the known species. Species from 12 genera within the Angraecoids, Vandeeae and the Orchidaceae were sampled as outgroups and to help the estimation of node ages on the phylogeny. Voucher specimens are held at BR, BRLU, K, NU, STPH and MO (herbarium acronyms follow Thiers, 2016). Based on an analysis of their morphological variation, we hypothesized the presence of eight new species in our samples (*Tridactyle* sp. 1, 2, 3, 4 on the continent and sp. A, sp. B, sp. C and sp. D on the islands) that will be described elsewhere (D'hajère et al., in prep).

### 2.2 | DNA extraction, genomic libraries and Illumina sequencing

Genomic DNA was extracted from  $\pm 0.03$  g of silica-gel dried leaf material and herbarium preserved specimens, using a DNeasy Plant Mini Kit™ following standard protocol (Qiagen Co.). DNA concentration and quality were evaluated using a Qubit® 2.0 Fluorometer (Life Technologies, Invitrogen) and a QIAxcel analyser (Qiagen). Approximately 1–2 µg of genomic DNA per sample was used for library construction. Genomic DNA was sheared by sonication using a Bioruptor® Pico (Diagenode SA.), to target a mean DNA fragments size of c. 400 bp per sample. The larger (>600 bp) and shorter (<200 bp) DNA fragments were removed using the AMPure XP protocol (Agencourt). Illumina libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs) following the manufacturer's protocol. Libraries were pooled equimolarly and subsequently sequenced using an Illumina NextSeq instrument at the GIGA platform. We sought 1 million paired-end reads (2 × 150 bp) per library.

### 2.3 | Chloroplast and ribosomal genome assembly, annotation and structure

After Illumina demultiplexing, paired-end reads were checked for quality control using FASTQC 0.11.5 (Andrews, 2010). Raw sequencing data were trimmed and filtered using TRIMMOMATIC 0.38 with a Phred quality score  $\leq 33$  (Bolger et al., 2014). A reference assembly for *Tridactyle* was created de novo using the GetOrganelle pipeline (default parameters for chloroplast or ribosomal; Jin et al., 2020). Plastome and nuclear ribosomal sequences were assembled for *Tridactyle tridactylites* (specimen Yaoundé Shadehouse 4878, BRLU) and will be used further as reference genomes. Several assemblies were performed using libraries with high number of reads and the aforementioned sample was selected among other ones, as assemblies for both sequences were better resolved comparatively. We mapped the trimmed reads of the remaining samples to these reference assemblies using the BURROWS-WHEELER ALIGNER (BWA mem 0.7.5) (Li & Durbin, 2010). The aligned reads were converted to a mpileup file using SAMTOOLS 1.9 (Cock et al., 2015; Danecek et al., 2011; Li, 2010, 2011; Li et al., 2009). Consensus calling (i.e. calling genotype for each position for each individual in the mpileup

file) was conducted using VARSCAN 2.3.7 (Koboldt et al., 2012) as in Migliore et al. (2019). The final dataset is a multi-fasta aligned matrix with missing, indel and heterozygote positions coded as N (i.e. to avoid nuclear copies of plastid regions and heteroplasmy (Scarcelli et al., 2016).

Genome annotation was performed using Geseq (Tillich et al., 2017) and manually curated using the software GENEIOUS 9.0.5 (Kearse et al., 2012). Visualization of the circular chloroplastic genome map of the *Tridactyle* was produced with OGDRAW (Greiner et al., 2019; Figure 1).

## 2.4 | Phylogenetic inference and divergence time estimation

We conducted separate phylogenetic analyses on a ribosomal dataset, a plastid dataset and on both datasets combined. Several samples were characterized by a significant number of missing sites and analyses were consecutively performed on two different versions of each dataset. Version 1 was inclusive, containing multiple samples per species when available (Appendix S1), while Version 2 included only one sample per species, the one with the minimum number of missing sites. This approach allowed us to mitigate the effect of missing data, as the second version of each dataset included only samples with the highest number of covered sites (in average, 83% of the positions are covered in the dataset). We used maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) to infer phylogenetic relationships among sequences. Substitution models and best partitioning scheme were selected for each dataset using ModelFinder (Kalyaanamoorthy et al., 2017) as implemented in IQ-TREE (Chernomor et al., 2016; Nguyen et al., 2015). The second-order information criterion (corrected Akaike information criterion) was used for model selection. Site heterogeneity was modelled using a gamma distribution. Parsimony analyses were run in PAUP\* 4.a165 (Swofford & Sullivan, 2009) through the CIPRES Science Gateway (Miller et al., 2011). All characters were unordered and equally weighted; gaps were coded as missing data. Heuristic searches were performed using tree-bisection-reconnection branch swapping and 1000 replicates with random-sequence addition, holding 10 trees at each step, and saving no more than 20 trees per replicate to reduce time spent swapping on large islands of trees. ML analyses were run with RAXML-HPC BLACKBOX 8.2.10 (Stamatakis, 2014) through the CIPRES Science Gateway (Miller et al., 2011). We estimated support via a bootstrap analysis using ML criterion implemented in RAXML (Stamatakis, 2014). The model available in CIPRES that was closest to those suggested by ModelFinder was GTR +  $\Gamma$  for all three datasets. BI analyses were carried out by running BEAST 2.6.2 (Bouckaert et al., 2019) on a computer cluster accessible through our university (<http://www.cec-hpc.be>). The models available in BEAST 2.6.2 that were closest to those suggested by ModelFinder are presented in Appendix S2. We performed two Monte Carlo Markov Chain (MCMC) runs in parallel of four chains each, for  $100 \times 10^6$  generations, with trees sampled every 5000 generations (Huelsenbeck &

Ronquist, 2001). BEAST output files were examined through TRACER 1.7.1 (Rambaut et al., 2018) to evaluate convergence, and ensure adequate effective sample sizes (ESS values) for all parameters. The Maximum Clade Credibility tree was then produced using TREEANNOTATOR 1.8 (Rambaut, 2007), selecting a burn-in of 25%, using the slow run option. Phylogenetic trees were visualized in FIGTREE 1.4.3 (Rambaut, 2007).

Estimating divergence times was conducted on the second version of the ribosomal and plastid datasets combined, to limit overestimation due to unequal sample sizes among species. The calibrated time phylogeny was obtained using the Bayesian MCMC inference implemented in BEAST 2.6.2 (Bouckaert et al., 2019). The analysis was done using the following priors: an uncorrelated lognormal relaxed clock model, which allows an independent optimization of mutation rates for each branch of the tree, a Yule process of speciation, which is adequate to analyse data at the interspecific level, and the substitution models and best partitioning scheme selected using ModelFinder (Kalyaanamoorthy et al., 2017).

Due to the lack of availability of orchid fossils close to the subtribe Angraecinae (Iles et al., 2015), three nodes were used as calibration points, one using a fossil outside the subtribe (primary calibration) and two others using previously estimated node ages (secondary calibrations). The primary calibration involved the fossil *Dendrobium winikaphyllum* Conran, Bannister & D.E. Lee of New Zealand (Conran et al., 2009). This fossil is placed approximately 23.0–20.0 Ma, and we used the prior 'Fossil calibration' as implemented in BEAST 2.6.2. The secondary calibrations were conducted using estimates from (Givnish et al., 2015), by setting the divergence date between Orchidoideae and Epidendroideae as a normal distributed random variable ( $M = 64.0$  Ma,  $SD = 2.0$ ) and the divergence date of the Angraecoids as a normal distributed random variable ( $M = 21.0$  Ma,  $SD = 2.0$ ). We performed two MCMC runs in parallel of four chains each for  $100 \times 10^6$  generations, with trees sampled every 5000 generations (Huelsenbeck & Ronquist, 2001). BEAST output files were examined through TRACER 1.7.1 (Rambaut et al., 2018) to evaluate convergence, and ensure adequate ESS (ESS values) for all parameters. The Maximum Clade Credibility tree was then produced using TREEANNOTATOR 1.8 (Rambaut, 2007), after a burn-in of 25%, using the slow run option. Phylogenetic trees were visualized in FIGTREE 1.4.3 (Rambaut, 2007).

## 2.5 | Ancestral areas estimation

*Tridactyle* occurs only in continental Africa and the GGI, while the outgroups occur in a variety of areas worldwide. We selected 14 geographical regions as character states, based on the bioregions recognized by Droissart et al. (2018), adding austral Africa and dividing the GGI in 3 regions since it is our studied area:

Austral Africa (A), Albertine Rift Mountain (B), Lower Guinea (C), Congolia (D), East African Montane (E), Zambesian (F), Upper Guinea (G), Bioko (H), São Tomé (I), Príncipe (J), Swahilian Coastal (L), Angola (M), Southern rift Montane (N) and rest of the world (K).

The distribution of each *Tridactyle* species has been checked on QGIS (QGIS.org, 2020) based on the RAINBO database (Dauby et al., 2016). Ancestral ranges were estimated using Reconstruct Ancestral State in Phylogenies software RASP 4 (Yu et al., 2020), which implements several statistical methods of ancestral reconstruction. Three methods available in RASP were used: Statistical Dispersal-Vicariance Analysis (S-DIVA) (Yu et al., 2010), Dispersal-Extinction-Cladogenesis model (DEC) and Bayesian binary method (BBM) (Yu et al., 2015). These analyses were applied on all posterior trees (10,000) allowing up to 7 areas per node.

### 3 | RESULTS

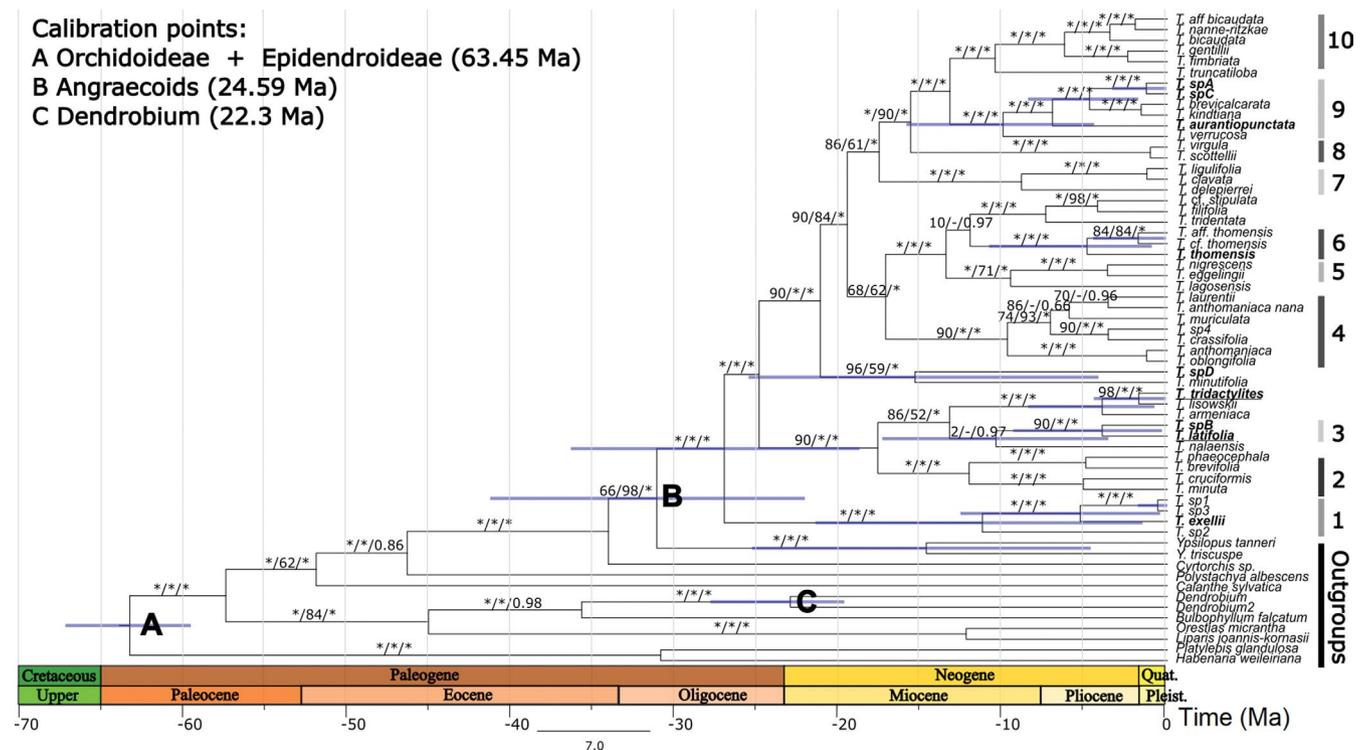
#### 3.1 | Molecular datasets and phylogenetic relationships

After read filtering, we obtained a mean of 1,074,057 good quality paired-end reads per sample ( $SD = 687,526$ ). The *T. tridactylites* plastid genome assembly was 154,624 bp in length, including a large single-copy region (LSC, 86,591 bp), a small single-copy region (SSC, 6911 bp) and a pair of inverted repeats (IR, 30,561) (Figure S1). The corresponding alignment contained 11,835 parsimony informative sites, for a total of 20,398 variable sites. Excluding the outgroups,

the number of parsimony informative sites dropped to 4994 for a total of 6972 variable sites.

The *T. tridactylites* ribosomal genome was 7212 bp in length, including an ETS + 18S region (2935 bp), a 28S region (3402 bp), a 5.8S region (164 bp), an Internal transcribed spacer 1 (ITS1) (228 bp) and an Internal transcribed spacer 2 (ITS2) (261 bp). The corresponding alignment contained 774 parsimony informative sites, for a total of 1174 variable sites. Excluding the outgroups, the number of parsimony informative sites dropped to 502 for a total of 726 variable sites. On average, 1.77% ( $SD = 0.29\%$ ) of trimmed reads were aligned to the plastome reference (mean number of reads = 19,296,  $SD = 3,198$ ). This corresponds to a mean depth coverage of 25x ( $SD = 23x$ , min = 0.8, max = 290). On average, 0.26% ( $SD = 1.2\%$ ) of trimmed reads were aligned to the ribosomal reference (mean number of reads = 2878,  $SD = 248$ ). This corresponds to a mean depth coverage of 132x ( $SD = 138x$ , min = 3.5, max = 719). For the second dataset (one sample per species), the chloroplast sequences had a mean depth coverage of 29x ( $SD = 23x$ ), and the ribosomal dataset a mean depth coverage of 84x ( $SD = 78x$ ).

The MP, ML and BI phylogenetic reconstructions obtained from all data combined for the second dataset, resulted in congruent topologies (Figure 2). Most relationships among clades were similar to those found in the phylogeny inferred from the plastid dataset and differed only in their poorly supported nodes (Figure S3.1). The



**FIGURE 2** Time-calibrated phylogenetic tree of *Tridactyle* and related genera, inferred with BEAST from the second version (one individual/species) of the combined plastid + ribosomal DNA dataset. 95% Highest Posterior Density of node heights are shown with blue bars for nodes discussed in the text. Letters A and B are calibration points from Givnish et al. (2015), C from Conran et al. (2009). Time scale is indicated below the tree. Bootstrap values from maximum likelihood analysis (first), bootstrap values from maximum parsimony analysis (second) and posterior probabilities (third) are shown above branches, \* represents 100 BS or 1 PP probabilities. Species names in bold letters are those found in the archipelago and those that are both in bold letters and underlined are found on both islands and the continent

phylogenetic analyses on the ribosomal dataset generated a less resolved tree, with mostly poorly supported nodes (Figure S3.2). However, the well-supported nodes were similar to the clades inferred from the plastid and combined datasets. The analysis of the combined dataset containing a single sample per species provided good support for relationships among *Tridactyle* species (Figure 2) while the analysis of the dataset containing all samples generated a similar topology, but with less supported nodes (Figure S3.3). The less resolved clades often contained samples with larger numbers of missing data.

In all, 20 species out of 34 were represented by more than one accession in the phylogeny containing all samples (Figures S3.1–S3.3). In most cases, conspecific samples (of valid or candidate species) formed a clade. However, for eight clades, conspecific samples did not form a monophyletic group (Figure S3.3). The last revision of the genus has been done in 1948 (Summerhayes, 1948), and multiple species have been added or put in synonymy since then; a taxonomic revision of the genus is probably necessary.

The following 10 clades within *Tridactyle*, which are morphologically cohesive, were supported by all analyses (Figures 2 and 3; Figures S3.1–S3.5): Clade 1, plants with short stem, comprising *T. exellii* and three probably new species from the CVL; Clade 2, plants with a gracile stem and inflorescences less than 4 cm, one group with leaves linear and short and the other with grass-like leaves, comprising 4 species from East Africa (*T. phaeocephala*, *T. brevifolia*, *T. cruciformis* and *T. minuta*); Clade 3, plants with oblong leaves, comprising *T. latifolia* and *T. spB*; Clade 4, plants with coriaceous leaves and short inflorescences, comprising *T. anthomaniaca*, *T. anthomaniaca* subsp. *nana*, *T. crassifolia*, *T. oblongifolia*, *T. muriculata*, *T. laurentii*; Clade 5, plants with thin short leaves and short inflorescences comprising *T. nigrescens* and *T. eggelingi*; Clade 6, plants with unindented (retuse) bilobed leaves and inflorescences of 1–4 cm long, comprising *T. thomensis* and two samples from Cameroon and Uganda, respectively; Clade 7, plants bearing stipules and flowers with a concave lip, comprising the species from the former genus *Eggelingia* Summerh. and *T. delepierrei*; Clade 8, plants bearing stipules and inflorescences of 1–4 cm long, comprising *T. scottellii* and *T. virgula*; Clade 9, plants with long inflorescences and lip with lateral lobes entire, comprising *T. verrucosa*, *T. aurantiopunctata*, *T. kindtiana*, *T. brevicealcarata*, *T. sp. A* and *T. sp. C* and finally Clade 10, plants with long inflorescences, lip with fimbriate sides lobes, comprising *T. bicaudata*, *T. nanne-ritzkae*, *T. gentillii* and *T. fimbriata*.

### 3.2 | Dating and biogeography

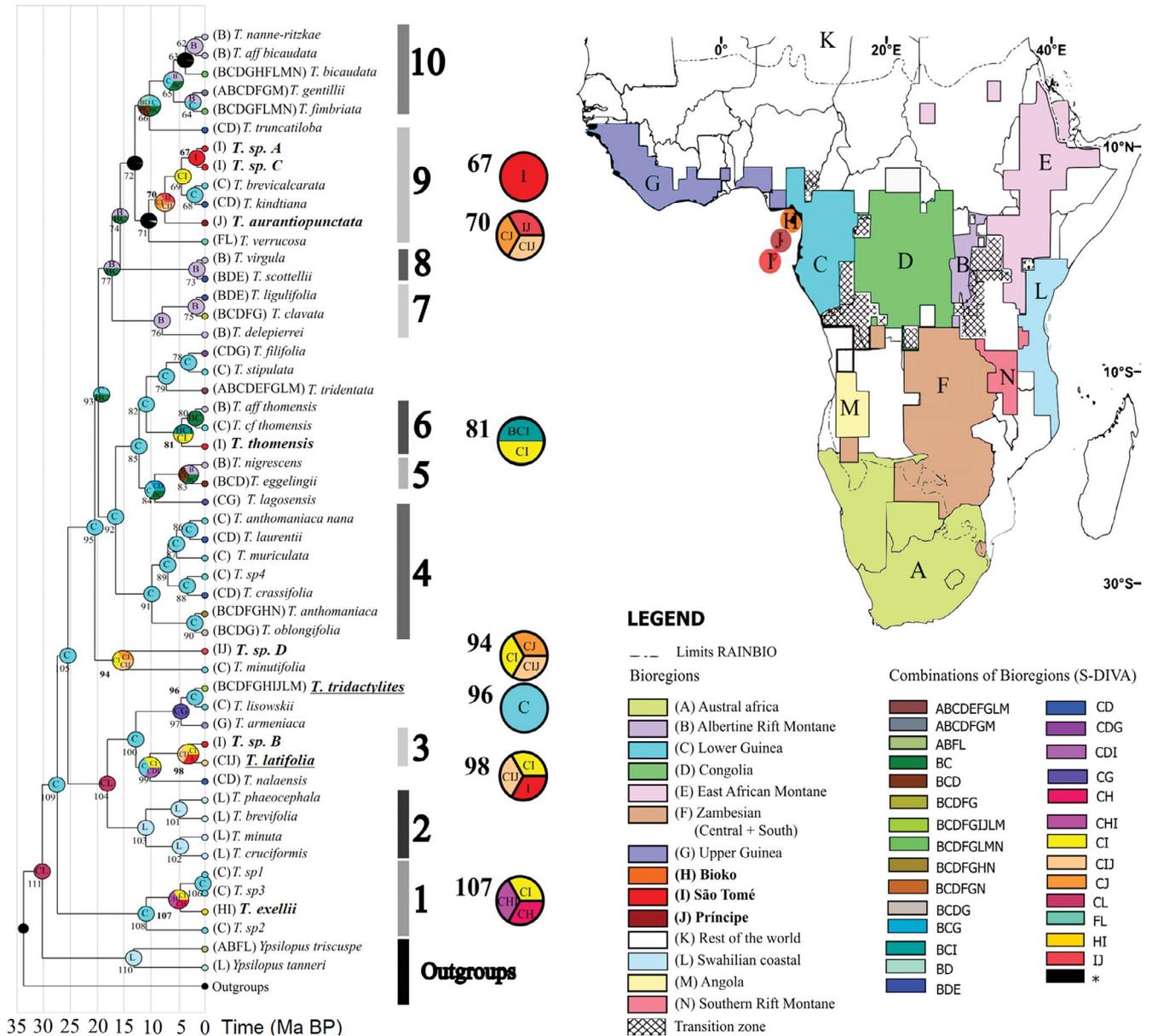
The phylogeny containing one sample per species (second dataset) was used for estimating divergence times and ancestral areas. The ancestral area reconstruction with S-DIVA and DEC was congruent when the probability was >50%, except for Clade 8 and node 111 (divergence *Tridactyle-Ypsilopus*) (Figure 3; Figure S3.6). BBM shows more mixed results, as most nodes are resolved at <50%,

reconstructions were congruent when the probability was >50%, except for Clade 4 and 8 and nodes 70, 93, 97 and 107 (Figures 3; Figure S3.7).

Divergence between *Tridactyle* and the other angraecoid orchids was estimated from all data combined for the second dataset, to have occurred during the lower Miocene, at 23.51 Ma (95% highest posterior density [HPD] = 19.97–27.24 Ma) (Figure 2). The ancestral area reconstruction with S-DIVA and DEC inferred the most probable region of the ancestor of *Tridactyle* as Lower Guinea (node 109, probability = 1.0) (Figure 3). The estimated ages of the ancestors of Clades 1, 2, 4, 7, 9 and 10 were all included in the late Miocene.

Using Figure 3, we can answer the three questions asked at the beginning of this study. First, the *Tridactyle* species of the GGI do not belong to a single clade, but are distributed in clades 1, 3, 6, and 9, and one species from São Tomé is grouped with another species from Gabon and Equatorial Guinea, *T. minutifolia*. Each island endemic species is more closely related to another continental species than to another species of the archipelago, with the exception of *T. spA* and *T. spC*. In total, we can infer at least six independent colonizations of the islands of São Tomé and/or Príncipe from the African continent. To answer our second question (did further diversification of the genus occur within the islands?), we can establish that evidence for speciation having occurred directly on the islands is scarce: the only strong evidence of a potential speciation event comes from the pair *Tridactyle spA* and *Tridactyle spC*. Finally, the only hint of a possible recolonization of the continent from the archipelago is given by ancestral node 70: its probability of having been located exclusively on the islands of São Tomé and Príncipe was estimated to be roughly 1/3, while this ancestral node is connected at the same time to a derived lineage that today is exclusively located on the continent. However, there is a larger probability (~2/3) that this ancestral species was located both on the islands and the continent. In that case, the current distribution pattern can also be explained by an initial colonization of the islands from the continent, followed by the disappearance of some descendant lineages from the continent or from the islands, depending on the species. As a consequence, our results show little evidence for recolonization of the continent from the islands.

Age estimates for ancestral nodes associated with a continental-island divergence leading to an island endemic species were as follows (Figure 3, from bottom to top of the tree): node 108: 13.57 Ma (95% HPD: 4.06–24.5 Ma) for *T. sp2* and *T. exellii* + *T. sp1* + *T. sp3*; node 107: 6.28 Ma (HPD: 0.88–14.37 Ma) for *T. exellii* and *T. sp1* + *T. sp3*; node 99: 10.66 (95% HPD: 0–16.8 Ma) for *T. nalaensis* and *T. latifolia* + *T. spB*; node 98: 4.05 Ma (95% HPD: 0–8.73 Ma) for *T. latifolia* and *T. spB*; node 96: 1.78 Ma (95% HPD: 0–4.18 Ma) for *T. tridactylites* and *T. lisowskii*; node 94: 15.67 Ma (95% HPD: 0–24.12 Ma) for *T. minutifolia* and *T. spD*; node 81: 4.99 Ma (95% HPD: 0–9.92 Ma) for *T. thomensis* and *T. cf thomensis* + *T. aff. Thomensis*; node 70: 10.22 Ma (95% HPD: 0–11.04 Ma) for *T. aurantiopunctata* and *T. spA* + *T. spC* + *T. brevicealcarata* + *T. kindtiana*; node 69: 4.84 Ma (95% HPD: 0–7.81 Ma) for *T. spA* + *T. spC* and *T. brevicealcarata* + *T. kindtiana*; and node 67: 1.31 Ma (95% HPD: 0–3.11 Ma) for *T. spA* and *T. spC*.



**FIGURE 3** Ancestral area reconstruction inferred with a Statistical Dispersal-Vicariance Analysis conducted on the estimated tree of Figure 2. Species names in larger font and bold letters are those found in the archipelago and those that are both in bold letters and underlined are found on both islands and the continent. Ancestral node of each pair of island species and their continental sister species are highlighted between the tree and the map. Pie charts at each node show probabilities of alternative ancestral ranges (if more than 2 alternative ranges, the node is repeated in larger size between the phylogeny and the map). Colours correspond either to a region indicated on the map or to a combination thereof; regions on the map, that are also identified by a letter, are bioregions previously identified by Droissart et al. (2018), with the addition of austral Africa and dividing further the Gulf of Guinea Islands in three separate regions: Bioko (H), São Tomé (I) and Príncipe (J); The colour black (asterisk) represents other ancestral ranges (not recovered by Reconstruct Ancestral State in Phylogenies). Different clades discussed in the Results and Discussion sections are highlighted by vertical bars to the right of the tree

## 4 | DISCUSSION

Our phylogenetic analyses provided a strongly supported phylogeny for the *Tridactyle* genus, which includes samples from herbarium specimens thanks to the use of NGS technology. Clade 2, that includes all species of the genus *Eggelingia*, is clearly embedded within *Tridactyle*, a

result already obtained by Simo-Droissart et al. (2018). The genus had already been placed as a section of *Tridactyle* by Rice (2005), which was confirmed by our study. Our biogeographical analysis suggested that *Tridactyle* arose in Lower Guinea, around 23 Ma.

Each island endemic species is more closely related to another continental species, instead of being all united in a single



monophyletic group, which would be expected under a scenario of island radiation. The pattern of diversification for the *Tridactyle* endemic species on the Gulf of Guinea islands suggests instead a polyphyletic origin and therefore, that the endemic lineages are the result of allopatric speciation, caused by the isolation of the island from the continent. It contradicts the assumption of Kisel and Barraclough (2010) that the presence of congeneric species on an island is unlikely the result of multiple independent colonizations, and the suggestion of Stuessy et al. (2006) that species radiation will more likely occur on mountainous islands associated with greater habitat diversity. This pattern inferred for *Tridactyle* in the Gulf of Guinea archipelago is different from the one retrieved for angraecoid orchids in the Mascarene archipelago (Micheneau, 2005; Micheneau et al., 2008), where it was inferred that a radiation event led to the appearance of five endemic species. Nevertheless, island colonization followed by allopatric divergence, but without subsequent island radiation, was often inferred in phylogenetic studies of several groups of plants in Macaronesia (Bramwell & Caujapé-Castells, 2011; Carine et al., 2004; Caujapé-Castells, 2004; Fernández-Mazuecos & Vargas, 2011; Jaén-Molina et al., 2021; Silvertown et al., 2005). The Mascarene islands are as mountainous as the Gulf of Guinea archipelago, but are far more distant from the closest large land mass, while Macaronesia is located at a similar distance than the GGI archipelago from the mainland. Our results are in line with the study of Rosindell and Phillimore (2011) that suggested that allopatric speciation following island colonization was the main model for islands located at an intermediate distance from the mainland, while species radiation occurring within islands was associated with greater distances from the continent.

The estimated ages of the ancestral nodes reuniting endemic lineages from the archipelago to lineages from the continent are all younger or at most as old as the estimated time of emergence of São Tomé, the youngest island. Plana et al. (2004), exploring the diversification of *Begoniaceae* in the archipelago, found a similar biogeographical pattern, in which the ages of nodes connecting island endemics to continental species were younger than the estimated ages of island emergence. It is therefore likely that these nodes represent independent colonizations of the islands from the continent, and are therefore neo-endemics. For *Tridactyle*, our estimates of the geographical range of the ancestral nodes implied a minimum of six independent colonizations of the GGI from continental Africa. This colonization could have occurred via the wind currents, either the African Easterly Jet at mid-tropospheric altitude or the austral winds from over the equator (Frédoux, 1994). Orchids are known to be capable of dispersal by wind over great distances, due to their dust-like seeds, comparable in size to bryophytes spores (Arditti & Ghani, 2000; Micheneau et al., 2008). However, the encounter of an appropriate mycorrhizal symbiont or of other specific plants (i.e. bryophytes) is needed for the germination and establishment of orchids (Dressler, 1981; García-Cancel et al., 2013). This constraint may explain that although multiple colonizations of the islands were inferred for *Tridactyle*, these remain exceptional historical events. If

recurrent migrations from the continent to the islands had occurred, we would not expect the presence of so many endemic species on these islands, as gene flow would have prevented allopatric divergence and speciation due to the isolation of the island from the continent.

Overall, we interpret the biogeographical pattern highlighted in *Tridactyle* as evidence that the isolation of the islands from the continent was the main driving force behind the appearance of new species on the islands, while intra-island speciation appears to have been limited. The general picture that emerges is thus one of multiple allopatric speciation events following island colonization. There may, however, be one exception to this general trend: *T. sp. A* and *T. sp. C*, within clade 9, have diverged recently from each other and are both endemics of São Tomé. They do not share a similar habitat: *T. sp. A* is found in secondary and old-secondary forest, primary evergreen mist-forest while *T. sp. C* is found only growing on *Cocos nucifera* L., in secondary habitats, near the sea. It is therefore possible that a speciation event occurred on the island through a process of habitat specialization. However, multiple individuals from each of these two species were not recovered as monophyletic groups on the tree inferred with version 1 of the three datasets (173 sequences; Figures S3.3–S3.5). Since our initial definition of these two species based on morphological variation was not confirmed by our analysis of molecular variation, more data will be needed before we can confirm the existence of these two species as well as the occurrence of a diversification event on the island. Note that evidence for speciation occurring on the GGI has already been reported for animal species, such as reed frogs (Bell, Drewes, & Zamudio, 2015) and freshwater crabs (Daniels & Klaus, 2018).

In general, the relative ambiguity of the estimated range of many ancestral nodes linking island species to continental species prevents us from drawing strong conclusions about inter-island colonizations or back-colonization from the GGI to the continent. Two species are found both on the archipelago and the continent, that is, *T. tridactylites* and *T. latifolia*. In both cases, this pattern suggests a relatively recent migration from the continent to the island or from the island to the continent. Inferring the direction of these migrations is important because it would allow us to test the hypothesis that islands can serve as a source of diversity for the continent. The data presented here are insufficient for such inference, but we plan to further explore this question by studying intra-species genetic variation.

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#### CONFLICT OF INTEREST

There is no conflict of interest.

#### DATA AVAILABILITY STATEMENT

The Herbarium de l'Université Libre de Bruxelles (BRLU), where the specimens used in this study were sent and are kept, is a scientific institute registered with CITES under the number BE-007. The chloroplast and ribosomal reference sequences are available on GenBank (accession numbers: MW760855 and MW760856) and all generated alignments are available on Dryad (<https://doi.org/10.5061/dryad.zkh189398>).

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**BIOSKETCH**

Tania D'hajjère is a PhD student interested in Evolutionary Biology, Systematics (Taxonomy) and Population Genetics. Her current project addresses the evolutionary and biogeographical processes underlying biodiversity patterns in tropical African plants. The experimental model, epiphytic orchids, is a prominent component of tropical rainforests, especially in São Tomé and Príncipe, two oceanic islands recognized as biodiversity hotspots. This work is part of her dissertation.

**Author Contributions:** TD, OH, TS and PM designed the project; TD, TS and BB collected the samples; TD conducted the laboratory work with the help of EK and AB, and performed all data analyses with the help of AB; TD and PM interpreted the data; TD and PM prepared the initial draft of the manuscript and all authors contributed to its revision and approved the final version.

**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher's website.

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