Running title: Origin and speciation of Orchids

THE ORIGIN AND SPECIATION OF ORCHIDS

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Summary [200 words]

- Orchids constitute one of the most spectacular radiations of flowering plants. However, their geographical origin, historical spread across the globe, and hotspots of speciation remain uncertain due to the lack of a broad phylogenomic framework.
- We present a new Orchidaceae phylogeny based on high-throughput and Sanger sequencing datasets, covering all five subfamilies, 17/22 tribes, 40/49 subtribes, 285/736 genera, and ~7% (1,921) of the currently 29,524 accepted species. We then use it to infer geographic range evolution, diversity, and speciation patterns by adding curated geographical distribution data through the World Checklist of Vascular Plants.
- Orchid's most recent common ancestor is traced back to the Late Cretaceous in Laurasia. The modern Southeast Asian range of subfamily Apostasioideae is interpreted as relictual, matching the history of numerous clades that went extinct at higher latitudes following the global climate cooled during the Oligocene. Despite their ancient origins, modern orchid species' diversity mainly originated over the last 5 Ma, with the fastest speciation rates found in south-eastern Central America.
- Our results substantially alter our understanding of the geographic origin of orchids, previously proposed as Australian, and further pinpoint the role of Central American as a region of recent and explosive speciation.

Keywords: Orchidaceae, historical biogeography, high-latitude extinction, macroevolution, Laurasia, Neotropics.

Main body [5521 words]

1. Introduction

The angiosperm tree of life is characterised by the rise and demise of species, leading to species-rich and depauperate lineages co-existing in time and space (**Magallón *et al.*, 2019; Tietje *et al.*, 2022**). Investigating factors behind angiosperm diversification requires ancient, species-rich clades thriving across the globe and supplemented with a fossil record. One such clade is the Orchidaceae. With 29,524 species (**Chase *et al.*, 2015**; **Christenhusz & Byng, 2016; Govaerts *et al.*, 2021**), orchids are among the most species-rich groups of flowering plants, with molecular-dating studies having estimated their initial diversification at 112-76 million years ago [Ma] (**Ramírez *et al.*, 2007; Gustafsson *et al.*, 2010; Chomicki *et al.*, 2015**; **Givnish *et al.*, 2016; Serna-Sánchez *et al.*, 2021**). This diversification time is partly supported by the orchid fossil record, which includes leaf compressions and pollinaria dated from the early Eocene through the mid-Miocene found in different deposits (**Ramírez *et al.*, 2007; Conran *et al.*, 2009; Poinar, 2016a, b; Poinar & Rasmussen, 2017**). Most of these fossils have been credibly assigned to subfamilies and tribes.

An updated biogeographic study of this old, species-rich, and cosmopolitan family (**Fig. 1a-d)**, which is uniquely diverse in pollination systems (**Ackerman *et al.*, 2023**) and adaptations to different habitats (Fig. **1e-f**), is an essential step towards a better understanding of monocot clades with wide distribution ranges and diversity of adaptations (e.g., Arecaceae [**Couvreur *et al.*, 2011**], Bromeliaceae [**Benzing, 2000**], Poaceae [**Soreng *et al.*, 2015**]). Such a study can build on several in-depth analyses that have focused on subclades (**Bouetard *et al.*, 2010**; **Guo *et al.*, 2012; Freudenstein & Chase, 2015; Pérez-Escobar *et al.*, 2017a; Nauheimer *et al.*, 2018**). In a benchmark study of the biogeographic history and diversification of the entire family, **Givnish *et al.* (2016**) used a fossil-calibrated plastid tree for 173 genera (out of 736 in total) of orchids representing all five subfamilies, along with selected outgroups in the Asparagales (Asteliaceae, Blandfordiaceae, Boryaceae, Hypoxidaceae, Iridaceae, Lanariaceae). Using a likelihood approach (**Matzke, 2013**) and a single consensus ultrametric tree, their biogeographic analyses relied on a geographic matrix where terminals, usually representing genera, were coded for the entire distribution of the respective genera. Their results pointed to an origin and initial diversification of orchids in Australia during the mid-Cretaceous, 120–90 Ma. Notably, their range estimates for the Orchidaceae stem node indicated Australia as ~40% likely, Neotropics plus Australia as ~20%, and all other ranges together as ~40%. At 90 Ma, Australia was at high latitudes connected to Antarctica and part of the southern supercontinent, Gondwana, with connections

to South America, and the authors, therefore, stressed the likely importance of expansion across Antarctica. A Gondwanan origin for Orchidaceae was also suggested by **Chase (2001)**. The extent to which taxon sampling, phylogenetic uncertainty, and the chosen approach for coding geographical ranges influenced the estimation of ancestral areas in Givnish *et al.*'s study remains unknown.

Givnish *et al.* (2016: Table 1) also addressed the correlates of diversification rates through time and inferred Southeast Asia as the region with the highest net diversification rates. Studying how diversification rates are linked to geographical variation helps understand not only the pace at which extant species diversity has accumulated but also the correlated biotic and abiotic variables (**Condamine *et al.*, 2013**; **Velasco & Pinto-Ledezma, 2022**). Although orchid species diversity is clearly unevenly distributed across their distribution (**Vitt *et al.*, 2023**), no study has yet assessed the relationship between the distribution of orchid species richness and their underlying current speciation rates at a global scale for the entire family.

Here, we revisit the biogeographic history of Orchidaceae and infer geographic patterns of speciation, using a greatly expanded taxon sampling (1,921 species, 285 genera) as compared to previous phylogenomic studies, with a particular focus on early divergences. We generate a robust phylogenomic framework by combining high-throughput and Sanger sequencing data. By employing multiple topologies as input for the phylogenetic comparative analyses, we account for the effects of branch length variation and uncertainty in the position of weakly supported lineages. Our spatial information derives from herbarium-vouchered, georeferenced occurrence data, sourced from the Global Biodiversity Information Facility (GBIF; https://www.gbif.org) and the RAINBIO mega-database (**Dauby *et al*., 2016**). This dataset was subsequently vetted using the spatial distribution data of the World Checklist of Vascular Plants (WCVP; **Govaerts *et al.*, 2021**). Based on these datasets, we: *i*) infer the evolutionary and biogeographical history of the orchid family, *ii*) test the hypothesis of an Australasian origin for the most recent common ancestor of living orchids, and *iii*) revisit the hypothesis that Southeast Asia is the region with the highest current diversification of orchids.

2. Material and Methods

2.1 Taxon sampling, DNA library preparation and sequencing

Our phylogenomic framework was assembled using a high-throughput-sequencing dataset, complemented with Sanger data for single genes. The high-throughput-sequencing

dataset focused on the genes targeted by the Angiosperms353 probe set (**Johnson *et al.*, 2019; Baker *et al.*, 2022**) and includes 448 orchid species, representing 285/736 genera, 40/49 subtribes, 17/22 tribes, and 5/5 subfamilies (in the classification of **Chase *et al.*, 2015**). These data were generated by the Plant and Fungal Trees of Life Project (**Baker *et al.*, 2022**) at the Royal Botanic Gardens, Kew, and the Genomics for Australian Plants Consortium (https://www.genomicsforaustralianplants.com/). Nineteen monocot species were included as outgroup taxa, with *Dioscorea caucasica* (Dioscoreales) used as a rooting terminal, for a total of 467 species in the high-throughput-sequencing dataset. Illumina sequencing reads were newly produced for 377 samples (*vs* **Pérez-Escobar *et al.*, 2021**) from expertly curated specimens housed in the Kew (K) and Australian National herbaria (CANB), including 23 species sampled from type material and silica-gel dried tissue (**Table S1**). Detailed protocols for DNA extraction and total genomic DNA libraries are provided in **Methods S1**.

The Sanger sequencing dataset was assembled through the SuperCRUNCH pipeline (**Portik *et al.*, 2020**). This pipeline was executed using an initial set of 24,172 sequences of the nuclear ribosomal ITS and the plastid *mat*K markers – two commonly sequenced, informative loci for Orchidaceae and other angiosperms (**Grace *et al.*, 2021**) – obtained from GenBank (https://www.ncbi.nlm.nih.gov/nuccore). The following parameters were used: *i*) search terms of 'ITS; ITS1; ITS2; internal transcribed spacer 1; partial sequence; 5.8S ribosomal RNA gene; complete sequence; internal transcribed spacer 2' for ITS, and '*matK*; *trnK*; maturase K; *trnK* gene, intron; and maturase K *matK* gene' for *matK*; *ii*) a list of 285 generic (accepted) names that were sampled in the high-throughput-sequencing dataset; and *iii*) similarity searches between sequences using MEGABLAST (**Camacho *et al.*, 2009**), retaining a maximum of 200 hits per search. After removing duplicated sequences and obvious contaminants (defined as sequences with misplaced positions given current taxonomic views), we retained sequences of nrITS and plastid *matK* for 2,060 species, of which 64% had sequences produced from the same voucher specimen (**Table S2**).

2.1 High-throughput and Sanger sequencing data analyses

Illumina libraries were quality-assessed with FastQC software v.0.11 (available at https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Paired-end reads were quality-filtered and adapter-trimmed using TrimGalore! V.0.6.4 available at (https://github.com/FelixKrueger/TrimGalore), using the following parameters: *i*) *-q 30* (minimum Phred score), *ii*) *–length* 20 (minimum read length), *iii*) retaining read pairs that passed the quality thresholds. In-silico retrieval of the Angiosperms353 coding loci was

conducted using the pipeline HybPiper v.2.1.6 (**Johnson *et al.*, 2016**) and the same parameters and software described in **Pérez-Escobar *et al.* (2021a;** also see *Methods S1*). Next, low-copy nuclear and Sanger markers were aligned using the software MAFFT v.7.4 (**Katoh & Standley, 2013**) in conjunction with the iterative refinement method FFT-NS-I, implementing a maximum of 1,000 iterations. To account for potential biases from missing data in phylogenetic analyses, sequences shorter than 50% of the total alignment length were excluded. Subsequently, alignments were filtered for misaligned positions using TAPER v.1.0 (**Zhang *et al.*, 2021**), using a cut-off value of 1 (flag –*c*), and subsequently inspected by eye with Geneious v.8.0 (available at https://www.geneious.com/). The Sanger/Angiosperms353 nucleotide alignments are accessible at (at10.6084/m9.figshare.22245940).

2.2 Distance-based, maximum likelihood, Bayesian, and multispecies coalescence phylogenomic inferences

We computed maximum likelihood (ML) trees from the individual Sanger and Angiosperms353 nucleotide alignments using RAxML v.8.0 (**Stamatakis 2014**) with the following parameters: *i*) 500 rapid bootstrap replicates (flags *-#* 500 and *-x*); *ii*) the GTR+ Γ nucleotide substitution model. Additionally, a multispecies coalescent (MSC) tree was inferred from the individual Angiosperms353 gene trees produced by RAxML using the software ASTRAL-III v.5.6 (**Zhang *et al.*, 2018**) and the flag –t 2 (full tree annotation), after collapsing bipartitions with a likelihood bootstrap percentage (LBP) < 20% (achieved with the function new_ed of the package *newick_utils* v.1.6.0, available at https://bioweb.pasteur.fr/packages/pack@newick-utils@1.6). To visualise the proportion of gene-tree quartets that agreed with the species tree, we produced quartet support pie charts for every branch represented in the species' tree, using the full annotation produced by ASTRAL-III. We also visualized the proportion of gene trees in agreement with each species' tree bipartition using Phyparts v.1.0, by labelling as informative any gene-tree bipartition with a LBP > 20% (**Smith *et al.*, 2015**).

Following the overall topological congruence amongst the ITS and *mat*K datasets and Angiosperms353 low-copy nuclear gene alignments revealed by the MSC and a goodness-of-fit test (**Balbuena *et al.*, 2013**; **Pérez-Escobar *et al.*, 2015**, see *Methods S1* and *Results and Discussion S1*), we also computed ML phylogenetic trees from *a*) a super-matrix derived by concatenating the ITS and *matK* sequences, *b*) a super-matrix derived from the concatenation of the Angiosperms353 loci using RaxML v.8.0 and the same settings previously specified,

considering each super-matrix as a single partition. Lastly, we inferred a consensus network using the 500 bootstrap replicates produced from the Angiosperms353 super-matrix by RAxML v8.0, using SplitsTree v.4.0 (**Huson & Bryant, 2006**), a mean edge weight, and a threshold value of 0.75 to filter out any split absent in such a proportion of the bootstrap trees. The alignments, trees, and consensus network are accessible at (at10.6084/m9.figshare.22245940).

2.3 Molecular clock dating analyses and species-level phylogeny assembly

Absolute age estimation analysis of orchid phylogenies was conducted in two stages, and by using a Bayesian framework implemented in BEAST v.2.6 (**Bouckaert *et al.*, 2019**), as follows:

(i) The backbone was inferred by subsampling the Angiosperms353 low copy nuclear gene alignments using SortaDate v.1.0 (**Smith *et al.*, 2018**). Here, we selected the top 25 low-copy nuclear gene alignments with the lowest root-to-tip variance coefficient (i.e., clock-likeness). This selection ensured the representation of the entire generic diversity as sampled by the ML high-throughput dataset (i.e., 285 genera; see *Methods S1*, and **Table S3, S4**). This data subset was imported in BEAUTi v.2.6 (**Bouckaert *et al.*, 2019**) as unlinked partitions, with the same priors employed by Pérez-Escobar *et al.* (**2021b**), as follows: *a*) the GTR nucleotide substitution model and a rate heterogeneity among sites modelled by a Γ distribution with four categories, *b*) an uncorrelated log-normal relaxed molecular clock in combination with a prior clock rate interval of 0.0001-0.001 substitutions/site/Ma, modelled by an uniform distribution, *c*) a birth-death tree process, modelled by a uniform distribution for the birth and relative death rates, *d*) three secondary calibration points retrieved from Givnish *et al.* (**2015**), modelled by normal distributions ($\sigma = 1$) and located at the root node, i.e., most recent common ancestor (MRCA) of Dioscoreales, Orchidaceae and Liliales (125 Ma), the MRCA of Orchidaceae (89 Ma) and the MRCA of subtribe Goodyerinae (32 Ma), *e*) the ML consensus phylogram of generic lineages produced in RaxML v.8.0 as a starting tree, *f*) 500 million generations, sampling every 100,000 generations and ensuring that all posterior values reached effective sample sizes (ESS) > 100. The proportion of informative and missing sequences per taxon sample is provided in **Table S4.** Finally, the support for the backbone ultrametric trees derived from BEAST was assessed by first computing a maximum

credibility consensus tree from the Markov chain Monte Carlo (MCMC) posterior trees using TreeAnnotator v.2.6 (https://www.beast2.org/treeannotator/), using a burn-in threshold of 10%, and then counting the proportion of bipartitions falling on different support intervals.

(ii) The species-level-ultrametric phylogeny of Orchidaceae was produced by inputting the ITS and *matK* alignments as unlinked partitions in BEAUTi v.2.6 (**Bouckaert *et al.*, 2019**), using the same priors described in (*i*). Furthermore, the ML consensus phylogram produced in RaxML v.8.0 from the ITS-*matK* supermatrix was used as a starting tree.

(iii) To account for phylogenetic uncertainty, we relied on constructing multiple ultrametric species trees through a novel pipeline (**Fig. 2**) as opposed to using a single consensus maximum clade credibility tree. We first randomly sampled 10 posterior trees derived from the BEAST analyses conducted on the genus-level Angiosperms353 and the ITS-*mat*K Sanger species-level datasets. Then, for each genus represented in the Angiosperms353 chronograms, we pruned its counterpart clade sampled on the species-level Sanger chronogram and proceeded to graft it onto the corresponding stem of the Angiosperms353 chronogram. This operation was conducted on each pair of randomly sampled posterior trees (hence called PP species trees). A detailed description of the pipeline is provided in the supplementary *Methods S1*.

2.4 Biogeographic analyses

Biogeographical state estimations were conducted on the 10 PP species trees. These were produced by pruning and grafting the Sanger ultrametric species trees onto the Angiosperms353 genus-level chronograms. We estimated areas of origin and geographic range using the ML approach of dispersal-extinction-cladogenesis (DEC, **Ree & Smith, 2008**) as implemented in the C++ version (**Beeravolu & Condamine, 2016**). The eight geographic areas considered were: (1) Palearctic, defined as Europe, Siberia, Central Asia, and Western Asia; (2) Nearctic, including all North America to the north of Tehuantepec in Mexico and excluding the southern tip of Florida; (3) Neotropics, including Central America, the Caribbean Islands, and South America; (4) Africa, defined as the whole African continent including Madagascar, and Arabian Peninsula; (5) Indomalaya, including India, southern China, and Wallacea; and (6) Australasia, defined as everything east of Heilprin-Lydekker's Line (**Ali & Heaney, 2021**). A time-stratified geographic model specified constraints on area

connectivity by coding 0 if any two areas are not connected or 1 if they are connected at a given period based on paleogeographic reconstructions. The first time-slice covers the Late Cretaceous and early Paleocene (100.5-60 Ma), corresponding to a time where Gondwana and Laurasia were already separated (**de Lamotte *et al.*, 2015**). The second time-slice covers the late Paleocene to early Oligocene (60–30 Ma), and the third covers the early Oligocene to the present (30-0 Ma). To avoid bias results supporting ancestral areas in favour of hyperdiverse biomes, our sampling per biogeographic area used proportional sampling that reflected known diversity patterns across biogeographic realms, with the Asian and American tropics hosting the highest levels of species richness (**Vitt *et al.*, 2023**). Here, we included 905 species distributed in the Neotropics (6% of total known diversity in the area; **Govaerts *et al.*, 2021**), 623 from the Indomalaya region (6%), 347 from the Palearctic (5%), 221 from the Afrotropics (9%), 180 from Australasia (15%), and 44 from the Nearctic (10%).

2.5 Spatial analysis of species diversity and speciation rates

We queried 29,524 accepted orchid species names obtained from the WCVP through the GBIF (https://www.gbif.org/) and RAINBIO (https://gdauby.github.io/rainbio/index.html; **Dauby *et al.*, 2016**) databases. Our initial step involved accessing the GBIF database through the R-package SPOCC (available at https://github.com/ropensci/spocc). Using the *occ* function, we downloaded up to 1,000 records for each queried species name, ensuring that we only selected records linked to geographical coordinates and preserved specimens. The RAINBIO repository was manually accessed. Next, we conducted an automated filtering using the SpeciesGeoCodeR package (**Töpel *et al.*, 2017**) to exclude duplicated records and those located within urban areas. Subsequently, we removed occurrences that fell outside global coastlines. Lastly, to mitigate the influence of misidentified records on downstream analyses (**Maldonado *et al.*, 2015**), we further filtered out records for which the distribution did not match with the species distribution per botanical country (level 3 of the World Geographical Scheme for Recording Plant Distributions; **Brummitt, 2001**) provided by the WCVP. The original record database sourced from GBIF is available at https://doi.org/10.15468/dl.v2gwxv, and the filtered geographical records used in all downstream analyses will be available at (*forthcoming FigShare link*).

Estimating net diversification rates (speciation [λ] minus extinction [μ]) in plant lineages remains challenging (**Louca & Pennell, 2021**). However, it is possible to estimate λ from a densely sampled and robust phylogenetic tree, if the provided sampling fraction included in that tree is known, and the phylogenetic framework is extensive (**Louca & Pennell, 2021**).

Our approach focuses on speciation dynamics, as inferred from tip λ rates. This metric represents contemporary rates of speciation for a given lineage and is less prone to bias than net diversification rates (**Title & Rabosky, 2019**). To obtain tip λ rates, we fitted a time-dependent model using BAMM v.2.5.0 (**Rabosky *et al.*, 2013**). This was informed by sampling fractions, contrasting accepted species number per genus from the WCVP database with the number of species sampled in our phylogenetic analyses. Here, 54% of the genera sampled attained a species representativeness of 10-50%, with 21% genera including sequences of > 50% of the known species diversity, and 25% sequences representing < 10% of the known species diversity. This analysis was conducted on each of the 10 PP species trees generated by pruning and grafting the genus- and species-level chronograms produced by BEAST. We initially computed prior values for the initial λ and shift parameters, using the R package BAMMtools (**Rabosky *et al.*, 2014),** and performed 10 million generations, sampling the MCMC simulations every 10,000 generations. The BAMM output was analysed in BAMMtools to ensure that all analyses reached convergence (ESS values > 100). Mean speciation rates at present time (λ tip rates) were extracted for all terminals of the phylogenetic trees using the *getTipRates* function in BAMMtools, and then linked to the filtered geographical species occurrences.

Global patterns of species richness were inferred by calculating the average number of species per political and botanical country, and per grid cell (100 km × 100 km), using the filtered occurrence records, as well as the average and maximum λ rate values per grid cell. All calculations were performed in the open source software QGIS v.3.0 (available at https://www.qgis.org/en/site/forusers/download.html) and the R package KEWR (Walker 2022) .

3. Results and Discussion

3.1. A new phylogenomic framework for the orchid family

This study underlines the advantage of combining high-throughput and Sanger sequencing datasets to achieve denser taxon sampling at relatively lower costs, while accounting for phylogenetic uncertainty. Our MSC and ML analyses yielded a well-supported phylogenomic framework for the Orchidaceae, containing 38% of the currently accepted orchid genera (Fig. **3**; **Fig. S1-S8; see Results and Discussion S1**). The relationships among subfamilies and tribes largely agree with findings from a recent study by **Zhang *et al.* (2023)**. This study presented a phylogeny of the Orchidaceae produced from 610 to 1,195 nuclear transcriptomes obtained from 437 species representing 131 genera. Notable exceptions

include the monophyly of Bletiinae within Epidendreae, and the position of Eriopsidinae within the Cymbidieae (Figure S2 in **Zhang *et al.*, 2023**). A detailed view of the summary trees produced by **Zhang *et al.*** reveals evidence of intragenomic conflicts (see Figure S3): 46 bipartitions in the MSC trees attained low local posterior probabilities (< 0.5), and LBPs (<70%), suggesting that dominant alternative relationships exist for the branches in question. Additionally, **Zhang *et al.*** recovered the subtribe Bletiinae as paraphyletic (i.e., *Bletia* and *Chysis* as successive sister to Ponerinae, Laeliinae and Pleurothallidinae), a relationship recovered with low support by **van den Berg *et al.* (2005)** and **Górniak *et al.* (2010**). Unfortunately, voucher information for the data of **Zhang *et al.* (2023)** has not been made publicly available (at the time of writing), which limits the extent to which these phylogenomic discrepancies can be evaluated (PRJNA923320 accession code, queried on 25 August 2023 in https://www.ncbi.nlm.nih.gov/, returned zero results).

Our results unambiguously place in the orchid tree of life the monospecific genus *Cooktownia* D.L.Jones (Orchidoideae) and the small genus *Claderia* (two species; Epidendroideae). These genera are distributed from the western Indian Ocean to the western Pacific and from the south-western Pacific to New Zealand. Previous classifications placed *Cooktownia robertsii* in the tribe Orchideae, based on stigmatic chamber characters (**Jones 1997**). Our findings suggest that *Cooktownia* is most closely related to *Habenaria* Willd., a large, almost cosmopolitan genus from subtribe Orchidinae. On the other hand, *Claderia* is confirmed as sister to *Agrostophyllum* Blume and *Earina* Lindl. (Agrostophyllinae, Epidendreae). We obtained sequences from the type specimen of *Claderia viridiflora* collected in 1867. Though the Angiosperms353 gene recovery was predictably low for this specimen, we successfully identified 40 genes, with 23 being informative (**Table S1**). *Claderia viridiflora* had been included in the predominantly African subtribe Eulophiinae based on morphological similarities in the gynostemium and unpublished nrITS sequences (**Cribb & Pridgeon, 2009**). Our placement in Agrostophyllinae agrees with **Niissalo *et al.*, (2023),** who recovered *Claderia* as a sister to *Agrostophyllum* based on plastid genomes and the nrITS loci, confirming the robustness of our findings.

3.2. *A Late Cretaceous, Laurasian origin of Orchidaceae and the relictual range of apostasioids*

The 10 PP species trees robustly supported relationships and revealed no major conflicts with the MSC tree produced in ASTRAL, with the sole exception of the position of *Corymborkis*, which, in some instances, was recovered as nested within tribe Gastrodieae

(**Fig. S7, S8**). The stem age of Orchidaceae is estimated to be around 120 ± 6 Ma (Fig. **S7**) and the crown age around 83 ± 10 Ma, corresponding to the Early and Late Cretaceous, respectively, in agreement with previous estimates (**Chase, 2001; Ramírez *et al.*, 2007**; **Chomicki *et al.*, 2015**; **Givnish *et al.*, 2016; Zhang *et al.*, 2023**). However, our results indicate that the majority of present-day orchid species diversity originated over the past 5 million years (Fig. **S9**).

Seven of the biogeographical estimates conducted on the PP species trees supported Laurasia (Nearctic + Palearctic) as the place of the initial diversification of extant orchids (relative probability = 0.15-0.30; **Table S5**), equivalent to their most recent common ancestor (Fig. **4a;** Table S5). Conversely, the three other estimates placed the orchid MRCA in Laurasia + Neotropics or in Gondwana (Neotropics + Australasia + Antarctica) (relative probability = 0.14-0.19). The second most likely areas had nearly equal probabilities to those for the first most likely area (relative probability = 0.14-0.18), all placing the orchid MRCA in Laurasia. This matches the important role that Laurasia played during the late Cretaceous, fostering the initial diversification of diverse tropical and warm temperate flowering plant lineages. Notable examples include the palm family Arecaeae (**Baker & Couvreur, 2013**) and the yams, *Dioscorea* (Dioscoreaceae: **Viruel *et al.*, 2016**).

During the late Cretaceous, Laurasian vegetation was dominated mostly by conifers, and angiosperms, whilst being diverse, were mostly herbs to small trees with early successional strategies restricted to unstable habitats (**Wing *et al.*, 1998; 2012**). The Laurasian Paleogene has a monsoon-influenced humid subtropical climate that supported closed canopy, broad-leaved deciduous angiosperm forests that extended into high paleolatitudes beyond the Arctic circle (**Eiserhardt *et al.*, 2017; Korasidis *et al.*, 2022**).

Proposing an initial diversification of the orchids in Laurasia contrasts with prior inferences of an Australian origin of the family, followed by entry into the Neotropics via Antarctica (**Fig. 4b**, **Givnish *et al.*, 2016**). **Givnish *et al.*** used nine species as representatives of astelid clades plus one species of Iridaceae, all in Asparagales, most of which are Australian. These outgroups may have influenced the estimate of the Orchidaceae stem node as Australia, for which a ~40% likelihood was inferred (with Neotropics + Australia inferred as ~20% likely, and all other ranges together as ~40%). Their range estimate for the Orchidaceae crown node was Australia ~20%, Neotropics + Australia ~30%, and all other ranges added together ~50%. Our broader sampling of orchid lineages and biogeographic analyses on multiple trees may lead to less phylogenetic and geographic uncertainty (**Rangel *et al.*, 2015**).

The subfamily Apostasioideae, sister to all other Orchidaceae and comprising only 16 species distributed in Southeast Asia and Northern Australasia (**Li *et al.*, 2013; Govaerts *et al.*, 2021**), is inferred by our study to have an ancestral occurrence in Australasia. This would mirror the ranges of numerous other plant groups that were widespread in the Eocene in Eurasia, but which survive only in tropical Southeast Asia today (**Manchester *et al.*, 2009; Meseguer & Condamine, 2020**). The pattern is known from other 50 groups of gymnosperms and angiosperms (mostly sclerophyllous plants) that formerly occurred in Europe and/or North America – the boreotropical flora of southern Laurasia (**Manchester *et al.*, 2009**). Descendants from this paleoflora survive today only in eastern Asia, which may have served as a late Cenozoic or Quaternary refugium for these taxa (**Manchester *et al.*, 2009**). An example amongst Orchidaceae is the subfamily Cypripedioideae (slipper orchids), with 169 extant species (136 Old World and 33 New World). **Guo et al., (2012)** estimated the ancestral range of this clade as Nearctic+Neotropics, in agreement with our study (**Fig. 4**).

The Indomalayan region harbours approximately 30% of the extant orchid species, mostly within the Epidendroideae. The oldest known orchid fossil is a hard epidendroid pollinarium found attached to a fungus gnat preserved in Baltic amber, dated to 55-40 Ma (**Poinar & Rasmussen, 2017**), documenting the presence of Epidendroideae at high latitudes in the early Eocene. During this period, evergreen forests covered northern Europe (**Collinson & Hooker, 2003**), and epiphytism likely had already evolved in epidendroid orchids (**Chomicki *et al.*, 2015**; **Collobert *et al.*, 2022**). Our biogeographic models, which excluded the Baltic amber pollinarium both as a fossil or as a geographic constraint, inferred that epidendroids were present in the Palearctic around 55–50 Ma, consistent with the fossil evidence. Hard pollinia are prevalent in Epidendroideae and probably appeared early in the history of the subfamily (e.g., in the MRCA of Sobralieae + remainder of epidendroids; **Dressler, 1990; Mosquera-Mosquera *et al.*, 2019**). However, the evolution of pollinia requires further study, integrating a broader phylogenetic framework in combination with pollinia trait data.

Subsequent colonisation of other biogeographical realms apparently occurred through long-distance dispersals, for example, from Indomalaya to the Afrotropics in tribe Vandeae (Epidendroideae) between 35-10 Ma, or through stepping-stone processes from the Palearctic and Nearctic to the Neotropics in tribe Cymbidieae (Epidendroideae, 30–20 Ma). In contrast, the early diversification of subfamily Orchidoideae probably took place 55-40 Ma between the Neotropics+Nearctic+Antarctic. This was followed by dispersals to Australasia and the Palearctic with subsequent *in-situ* Neotropical diversifications (e.g., Cranichidinae), Nearctic

(Spiranthinae), Paleartic, Indomalaya (Goodyerinae) and Afrotropics (e.g., Disinae) (**Data S1**).

3.3 A Central American hotspot of orchid speciation

In total, 795,735 records were downloaded from the GBIF and RAINBIO repositories. After rigorously filtering out duplicate records and inaccurate distributions *sensu* the WCVP database (see *Methods*), 495,755 accessions were retained. Analyses of political country species richness indicated that Ecuador, Colombia, and Papua New Guinea are the top three countries in terms of species-richness. Notably, seven out of ten most species-rich countries are located in the Neotropics. Analyses based on the botanical country species richness (as inferred from the WCVP) yielded similar results (**Fig. 5a** [inset], **Fig. S10, Table S6**). An analysis of species richness per grid cell derived from the curated GBIF-RAINBIO dataset, showed that Central America (especially Costa Rica) and the northern Andean region (particularly Ecuador and Colombia) have the highest levels of species richness. These geographical patterns of species richness patterns are thus in agreement with the species richness distributions independently obtained through the WCVP database and support findings of studies conducted at the family level (**Vitt *et al.*, 2023**) and in the Orchidoideae (**Thompson *et al.*, 2023**).

Geographical speciation (λ) patterns in orchids, as informed from tip λ rates, did not always coincide with the areas of highest current species richness. This discrepancy supports, in some cases, the findings of a recent study on global plant diversification rates and species richness patterns (**Tietje *et al.*, 2021**). Contrary to the findings of Pérez-Escobar *et al.*, (**2017a**), the northern Andes do not appear to host the fastest speciating orchid clades in the American tropics despite being one of the most species-rich areas worldwide (**Pérez-Escobar *et al.*, 2022**). Our findings suggest that southern Mesoamerica, comprising the moist and seasonal forests of Costa Rica and Panama, has the highest speciation rates per grid cell, a region that also exhibits outstandingly high levels of species richness (**Myers *et al.*, 2000; Mittermeier *et al.*, 2011; Crain & Fernandez, 2020**, **Fig. 5c; Table S7**). Notably, when considering minimum and maximum values of speciation rates, virtually all ecoregions assessed here presented one- or two-fold variation in their rate (**Fig. 5c, inset**).

Unlike the observed congruence between speciation patterns and species richness throughout most of the Neotropical regions, our study also revealed that southern Australia recovered lower λ tip rates (**Fig. 5c**), even though it shows high levels of species richness (**Myers *et al.*, 2000**). This implies that elevated species richness in the region might have

accumulated at much slower pace and over longer time scales. Whether specific Australian lineages contradict this general pattern remains to be assessed, a task that will require substantially larger sampling. While most of the orchid diversity in southern Australia is terrestrial (**Ackerman, 2019**), a trait associated with lower speciation rates (**Givnish *et al.*, 2015**), a direct link between high species richness and speciation rates appear to occur in the predominantly terrestrial Orchidoideae (e.g., *Habenaria*; **Thompson *et al.*, 2023**).

Our speciation rate analyses unveiled multiple accelerations across all subfamilies (**Data S1**). Notably, the majority of these accelerations were nested in the Vanilloideae, Cypripediodeae, Orchidoideae and Epidendroideae subfamilies. Within these, the shifts leading to the fastest tip rates occurred in the Orchidoideae and Epidendroideae, starting from the early Miocene (**Fig. 6a**). These shifts coincided with the initial diversification of species-rich genera such as *Maxillaria and Dendrobium*. In other instances, however, rate increases preceded the divergence of species-rich and depauperate clades as seen with *Lepanthes and Lepanthopsis* or occurred within genera like *Bulbophyllum and Habenaria*. Remarkably, the lineages with the highest speciating lineages are Neotropical epiphytes, including Epidendreae, Maxillariinae, and Oncidiinae. The sole exceptions to this trend are the nearly cosmopolitan terrestrial genus *Habenaria* (**Batista *et al.*, 2011**) and the genera *Bulbophyllum* and *Dendrobium*, which are mostly epiphytic and distributed mainly in tropical Asia (**Fig. 6b**, **Xiang *et al.*, 2016; Simpson *et al.*, 2022**). These findings are in line with previous macroevolutionary studies conducted for the entire family (**Givnish *et al.*, 2015; Zhang *et al.*, 2023**) or focused on specific lineages such as Cymbidieae and Pleurothallidinae (**Pérez-Escobar *et al.*, 2017**) or Orchidinae (**Lai *et al.*, 2021**).

Overall, the most rapidly speciating lineages predominantly occur in south-eastern Central America. These lineages span diverse habitats, from lowland dry and wet forests, through cloud forests, to high-elevation grasslands, as exemplified by genera like *Epidendrum*, *Lepanthes*, *Maxillaria*, *Pleurothallis*, and *Stelis* (Fig. **6a**). Interestingly, Central American cloud forests and high-elevation grasslands are of Pliocene origin, making them some of the youngest Neotropical biomes (**Kirby, 2011**). The Late Cenozoic shallow subduction of the young hotspot Cocos ridge under the Panama block (part of the Caribbean plate) is controlling the rapid uplift of the Cordillera de Talamanca in southeast Costa Rica and eastern Panama (**Morell *et al.*, 2011**). This ongoing process probably started between 5.5 – 3.5 Ma (**Grafe *et al.*, 2002**), with high elevated mountain uplift rates of ~1 m per 1 Ma (**Driese *et al.*, 2007**). The rapid creation of topography within a tropical climate could generate multiple microhabitats that might have enhanced speciation rates. Additionally, the

geographic position of Costa Rica and Panama acts as a biological crossroads between the two biodiversity hotspots of northern Mesoamerica and the northern Andes, resulting in the current coexistence of northern and southern lineages (**Burger, 1980; Kapelle, 2016**). These findings support and expand the hypothesis of Central American tropical forests having evolved rapidly and recently (**Cano *et al.*, 2022**). Nevertheless, although our analyses of geographical speciation rates offer new perspectives on the evolution of orchid diversity, we urge caution in interpreting the relationship between λ and species richness (Tietje et al., 2021). Additionally, our taxon sampling and species-level spatial distributions are likely biased due to uneven collection efforts in certain regions, such as the historically understudied New Guinea (**Camara-Leret *et al.*, 2020**).

4. Conclusions

This study places the initial diversification of orchids in Laurasia during the Late Cretaceous (83 ± 10 Ma) and infers today's geographic range of Apostasioideae as relictual. The presence of Orchidaceae in Eurasia and North America during the Paleogene, followed by extinction and survival in more southern regions, matches the historical patterns supported by the fossil records of at least 50 genera of other families that were once diverse in Eurasia, but today have smaller surviving groups in tropical Southeast Asia. We reject the hypothesis that Southeast Asia is the region with highest speciation rates (**Givnish *et al.*, 2016).** Instead, our results show that southern Central America, which contains 4.5% of the world's flora and fauna in just 0.5% of its land surface, shows the highest speciation rates per grid cell on a global scale. Moreover, these biomes have served as a hotspot for orchid speciation since the Pliocene. Future work could aim at increasing the species-level sampling in phylogenomic trees, particularly for regions and clades currently undersampled. Such additions will further our ability to infer patterns and processes of diversification at increasingly finer spatial and temporal scales.

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7. Author contributions

Conceptualisation: OAPE, DB, SSR, AA, WJB. **Methodology:** OAPE, FC, DB, SB. **Data curation:** OM, LS, NASP. **Resources**: OAPE, NASP, AZ, DB AQH, BC, KN, MAC, LL. **Software:** OAPE, FC, SB. **Investigation:** NASP, AQH, KA, LS, AZ, LL. **Formal analysis:** OAPE, FC, DB, SB. **Funding acquisition:** WJB, FF, IJL, AA, KN, MAC, OAPE, RPB, RM. **Visualisation:** OAPE, DB, MC, LO, SB. **Writing – original draft:** OAPE, SSR. **Writing – review & editing:** OAPE, SSR, CJ, AA, MC, JA, RT, FLC, DB, NASP, WJB, with further contributions from all co-authors.

8. Data availability

The data that support the findings of this study are openly available in https://treeoflife.kew.org/specimen-viewer and at10.6084/m9.figshare.22245940

Legends

Figure 1. Diversity of orchid habitats (A-C), modern distribution range (D) and pollination systems in Orchidaceae (E-G). **A**) Tropical cloud forests; **B**) Tropical lowland wet forests; **C**) High-elevation grasslands; **E**) the Neotropical epiphyte *Cycnoches guttulatum* Schltr., pollinated by male euglossine bees collecting aromatic compounds; **F**) *Trigona* bee carrying a pollinarium of a Neotropical *Xylobium elongatum* (top: dorsal view; bottom: side view); G) the epiphytic and leafless *Dendrophylax sallei* (Rchb.f.) Benth. ex Rolfe., a Neotropical orchid pollinated by moths. Photos: Oscar A. Pérez-Escobar & Diego Bogarín.

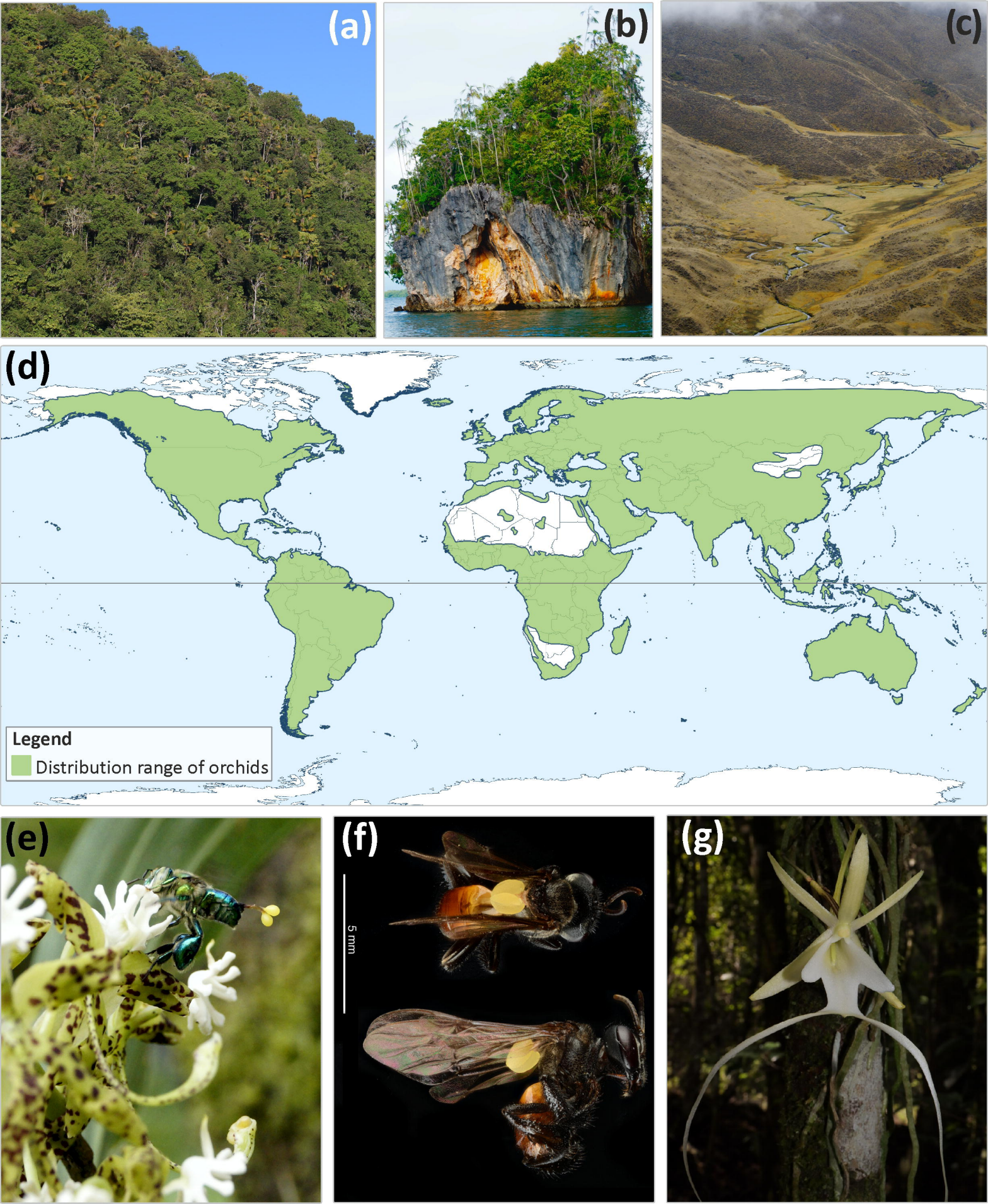
Figure 2. An overview of the pipeline used to produce ultrametric NGS-Sanger merged posterior probability species trees (PP species trees). The pipeline uses as input a custom set of randomly sampled MCMC trees derived from independently conducted molecular clock dating analyses on the genus-level Angiosperms353 and species-level Sanger gene alignments. It then produces a custom set of species-level trees that reflect branch length variation and support in deep (tribe, subtribe and genus) and recent (species) time as informed by the Angiosperms353 and Sanger datasets. A detailed description of the pipeline is provided in the supplementary *Methods S1*.

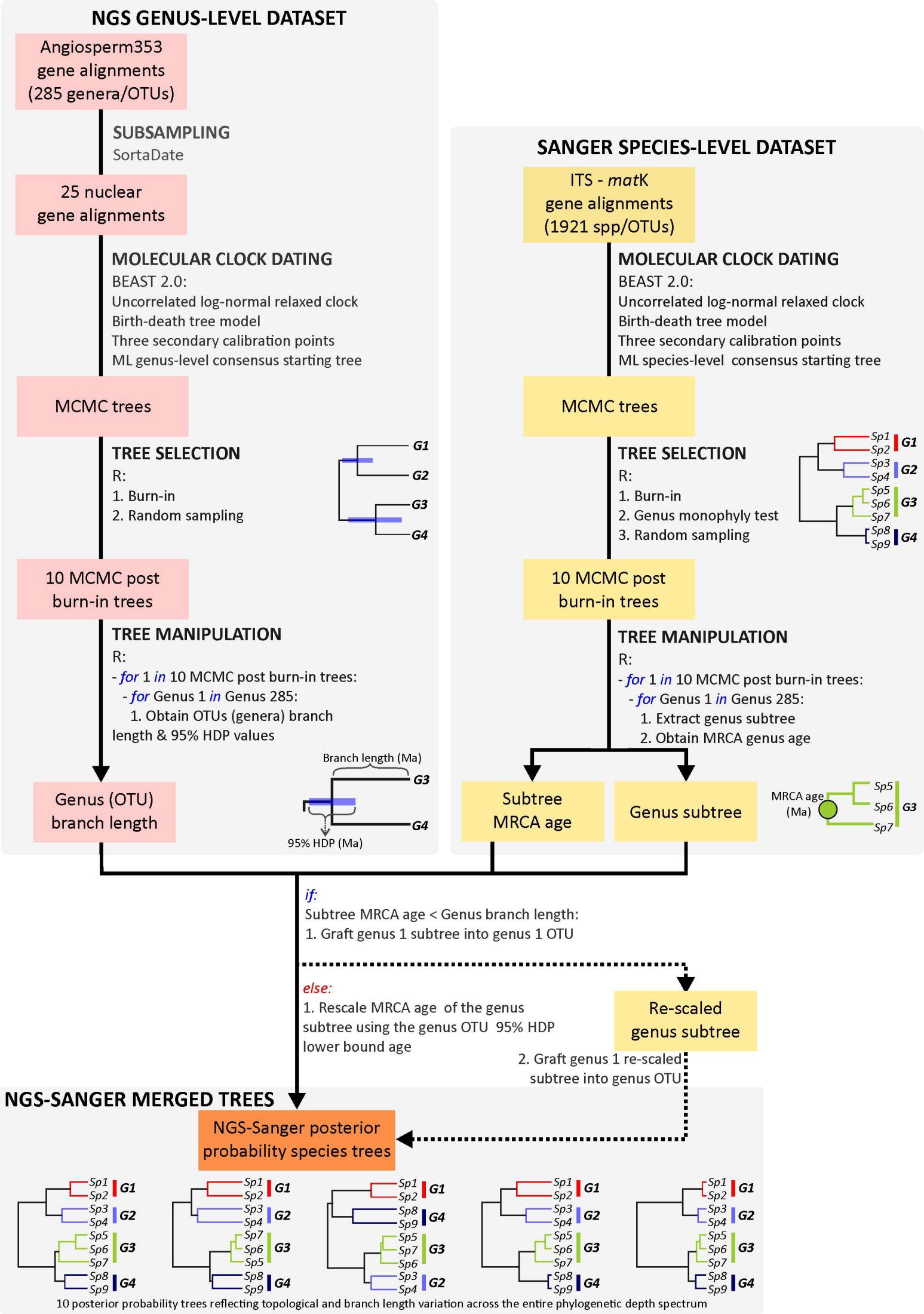
Figure 3. Phylogenetic relationships of Orchidaceae. **A**) Consensus tree-network inferred from 200 bootstrap replicate maximum likelihood (ML) trees derived from the concatenated alignment of 339 low-copy nuclear genes. Circles at nodes represent bipartitions present in > 75% of the bootstrap ML trees (see Figures S1-S6 for a detailed on the consensus tree-network). Non monophyletic groups are highlighted in bold and grey. Samples sequenced from typological material are highlighted in bold and pink. **B**) Number of bipartitions (bp) with different likelihood bootstrap support (LBS) values derived from an ML tree inferred from the 339 low-copy nuclear gene alignment. **C**) Number of bp with different LBS values derived from 339 nuclear ML gene trees. Grey bars represent poorly supported bipartitions, whereas yellow bars represent moderately to maximally supported bipartitions. **D**) Normalised Q-score value derived from an ASTRAL-III analysis, computed from 339 nuclear ML gene trees. Photos: Oscar A. Pérez-Escobar, Diego Bogarín, Sebastian Vieria, Kerry Dressler.

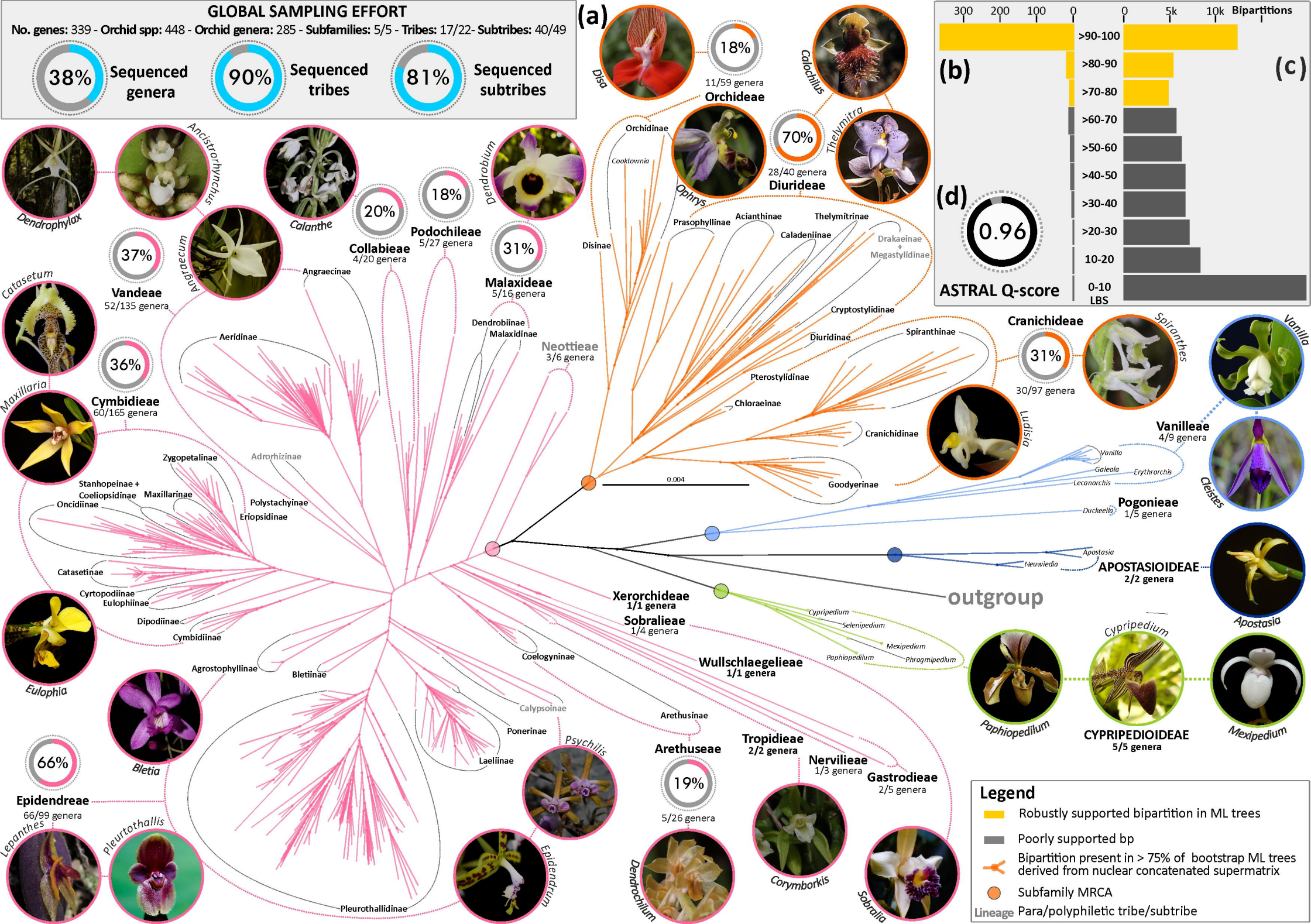
Figure 4. Biogeographic history of the orchid family. A) Ancestral areas at nodes inferred on the ten posterior probability species trees as estimated by a DEC model (see *Methods*) and summarised to the five orchid subfamilies (Inset: A summary of the outgroup sampling considered in our study (the MRCA of orchids is indicated with a black star). B) A palaeoclimatic and tectonic plate reconstruction at 90–80 Ma from **Burgener et al., 2023** showing the possible ancestral range of the orchid MRCA as estimated by **Givnish et al., 2016** and this study. A detailed account of the first and second most likely ancestral areas estimated to the tribe level is provided on Table S5 (complete results including annotated trees with the most likely ancestral area at nodes and alternative ancestral areas, likelihoods and probabilities are available at 10.6084/m9.figshare.22245940).

Figure 5. Geography of speciation and species diversity of Orchidaceae. A) Global patterns of species richness per grid-cell (100 ×100 Km), calculated from a curated database of geographical distribution records; reddish colours indicate higher numbers of species per grid-cell whereas bluish colours indicate lower numbers of species per grid-cell. A map of species richness per botanical country is provided in **Figure S10.** (Inset: orchid species numbers reported for the 10 most orchid biodiverse countries). B) Global patterns of mean λ tip rates (linear scale) per grid-cell (100*100 Km) as derived from the BAMM software; warm colours indicate higher λ tip rates grid-cell whereas cold colours indicate lower λ tip rates (Inset: a histogram of the mean λ tip rates attained across all grid cells). C) Global patterns of mean λ tip rates per ecological region, as defined by the WWF, derived from the BAMM software; warm colours indicate higher numbers of mean λ tip rates per ecological region whereas cold colours indicate lower mean λ tip rates. The highlighted geographical areas indicate the five ecoregions with the highest mean λ tip rates (Inset: Maximum and minimum λ tip rate values for ecoregions containing 100 or more geographical distribution records for which λ tip rates were linked).

Figure 6. Speciation dynamics of Orchidaceae. A) One of ten posterior probability species trees with modelled speciation (λ) rates along branches (blue colours denote low λ rates, reddish colours indicate high λ rates). Small circles at nodes denote the most recent common ancestors (MRCA) of orchid tribes sampled in this study. Numbers at nodes indicate the lineages with the highest λ tip rates across the entire family. B) Tip λ rate values (maximum, minimum, mean) derived from the ten posterior probability (PP) species trees for all terminals sampled. The dots are colour-coded by taxonomic groups (tribes and subfamilies). (Inset: λ tip rates summarised from the ten PP species trees for the genera associated to the highest λ tip rates [their respective totals of accepted species are provided]. Boxplots are colour-coded by tribes).

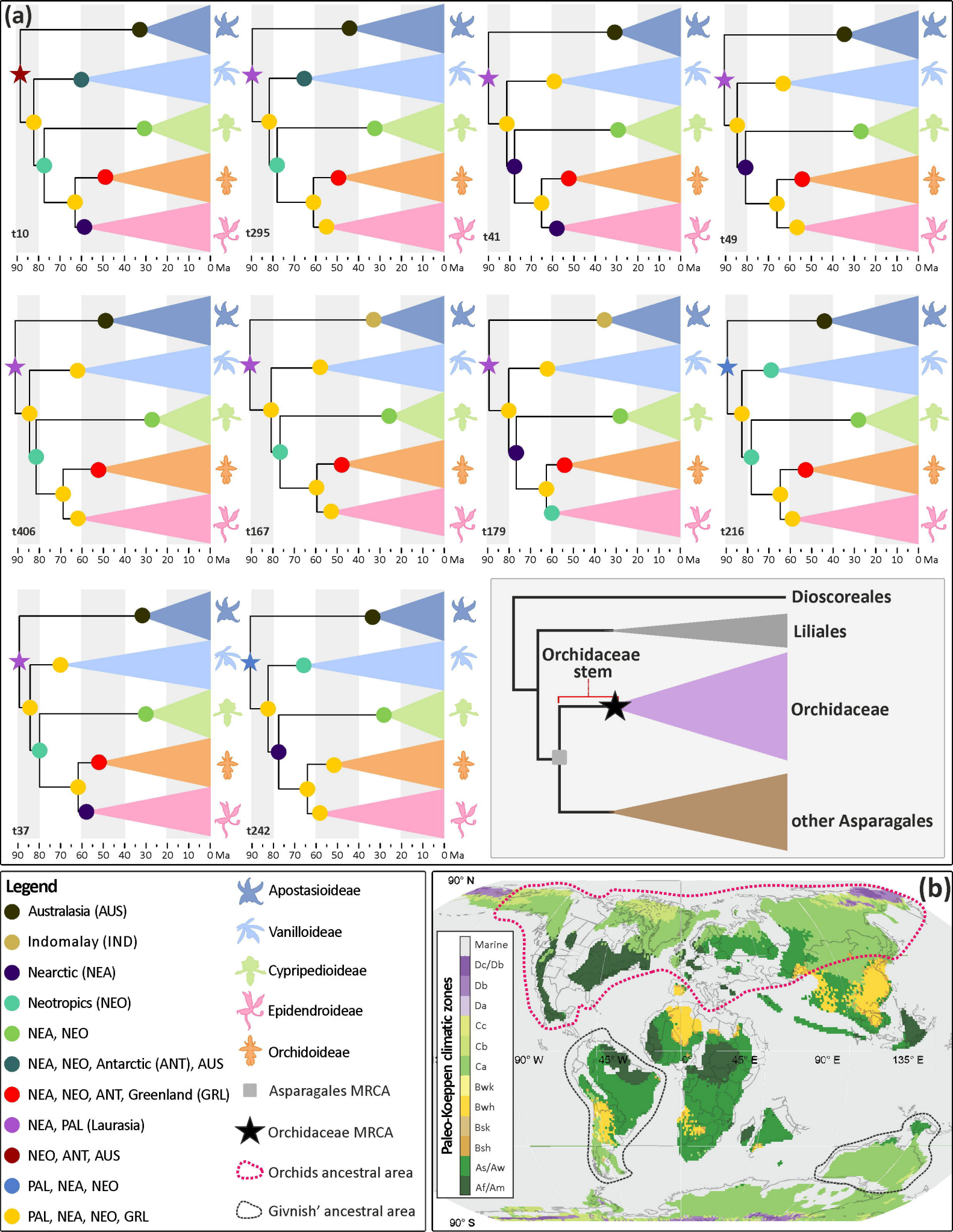

(a)
(b)
(c)
(d)
Legend
Distribution range of orchids
(e)
(f)
5 mm
(g)

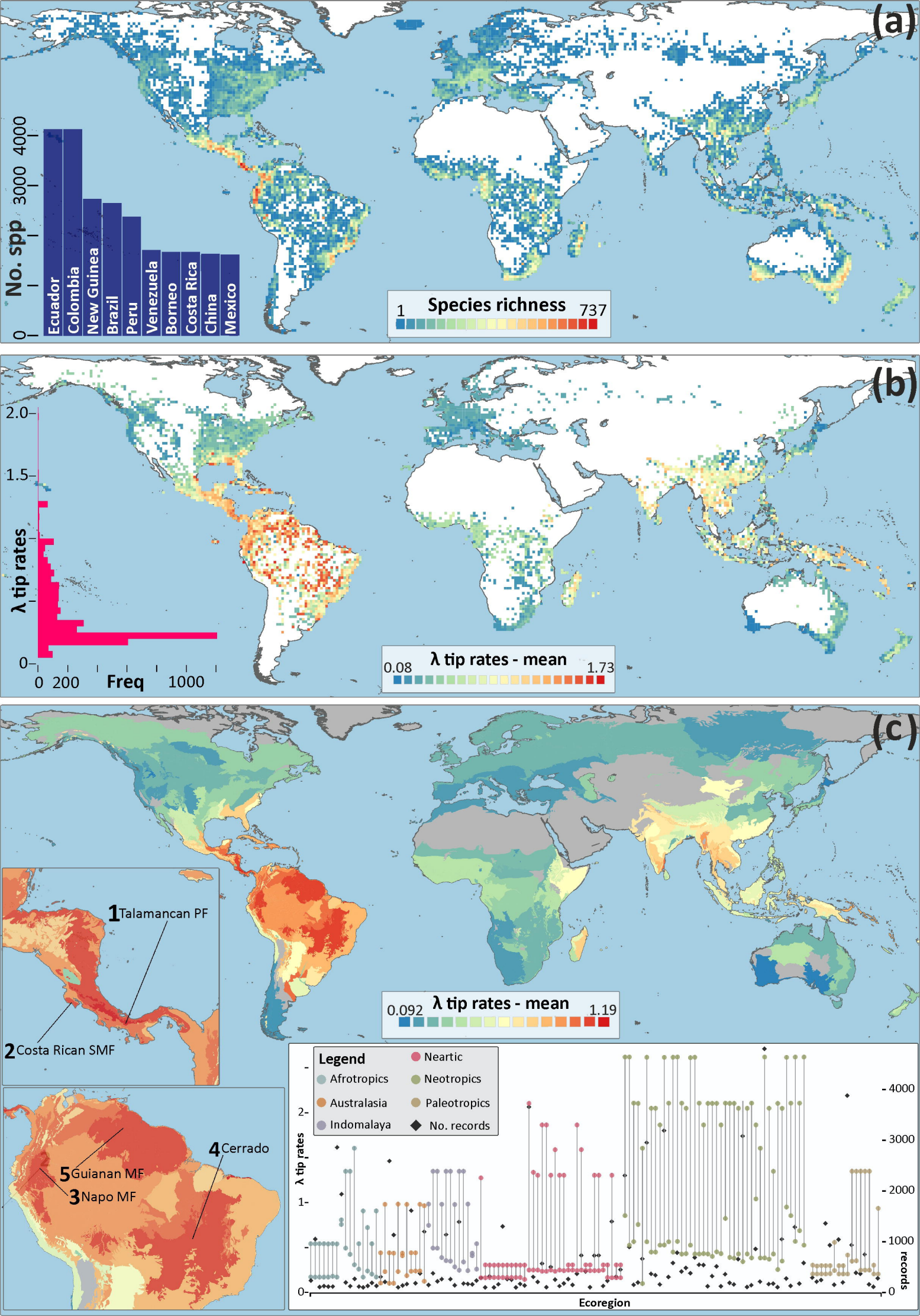

NGS GENUS-LEVEL DATASET
Angiosperm353 gene alignments (285 genera/OTUs)
SUBSAMPLING
SortaDate
25 nuclear gene alignments
MOLECULAR CLOCK DATING
BEAST 2.0:
Uncorrelated log-normal relaxed clock
Birth-death tree model
Three secondary calibration points
ML genus-level consensus starting tree
MCMC trees
TREE SELECTION
R:
1. Burn-in
2. Random sampling
G1
G2
G3
G4
10 MCMC post burn-in trees
TREE MANIPULATION
R:
- for 1 in 10 MCMC post burn-in trees:
- for Genus 1 in Genus 285:
1. Obtain OTUs (genera) branch length & 95% HDP values
Branch length (Ma)
95% HDP (Ma)
Genus (OTU) branch length
SANGER SPECIES-LEVEL DATASET
ITS - matK gene alignments (1921 spp/OTUs)
MOLECULAR CLOCK DATING
BEAST 2.0:
Uncorrelated log-normal relaxed clock
Birth-death tree model
Three secondary calibration points
ML species-level consensus starting tree
MCMC trees
TREE SELECTION
R:
1. Burn-in
2. Genus monophyly test
3. Random sampling
Sp1 Sp2 Sp3 Sp4 Sp5 Sp6 Sp7 Sp8 Sp9
10 MCMC post burn-in trees
TREE MANIPULATION
R:
- for 1 in 10 MCMC post burn-in trees:
- for Genus 1 in Genus 285:
1. Extract genus subtree
2. Obtain MRCA genus age
Subtree MRCA age
Genus subtree
MRCA age (Ma)
if:
Subtree MRCA age < Genus branch length:
1. Graft genus 1 subtree into genus 1 OTU
else:
1. Rescale MRCA age of the genus subtree using the genus OTU 95% HDP lower bound age
Re-scaled genus subtree
2. Graft genus 1 re-scaled subtree into genus OTU
NGS-SANGER MERGED TREES
NGS-Sanger posterior probability species trees
10 posterior probability trees reflecting topological and branch length variation across the entire phylogenetic depth spectrum

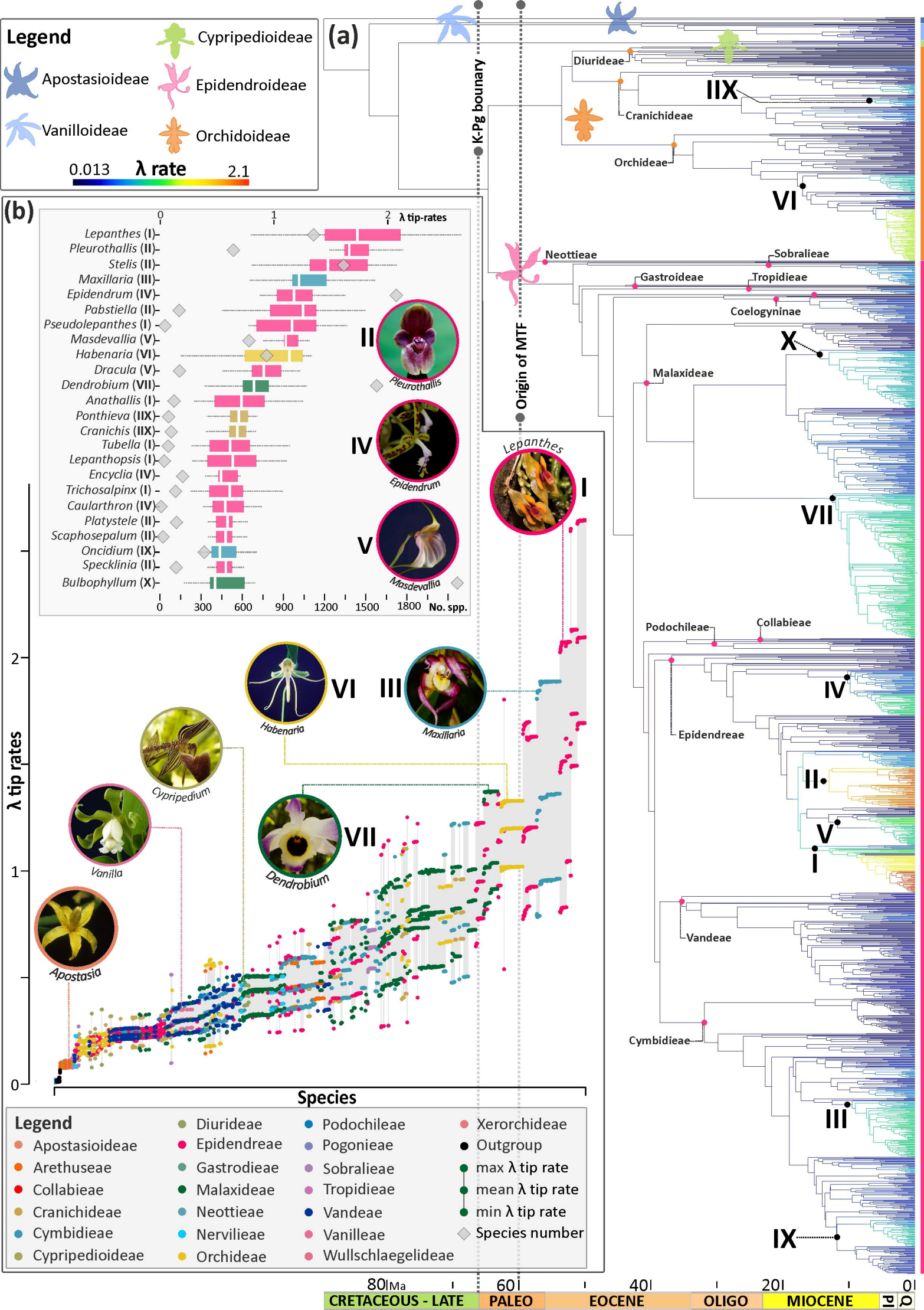


Legend

- Robustly supported bipartition in ML trees
- Poorly supported bp
- Bipartition present in > 75% of bootstrap ML trees derived from nuclear concatenated supermatrix
- Subfamily MRCA
- Lineage Para/polyphiletic tribe/subtribe


(a)
t10
t295
t41
t49
t406
t167
t179
t216
t37
t242
90 80 70 60 50 40 30 20 10 0 Ma
Dioscoreales
Liliales
Orchidaceae stem
Orchidaceae
other Asparagales
Legend
Australasia (AUS)
Indomalay (IND)
Nearctic (NEA)
Neotropics (NEO)
NEA, NEO
NEA, NEO, Antarctic (ANT), AUS
NEA, NEO, ANT, Greenland (GRL)
NEA, PAL (Laurasia)
NEO, ANT, AUS
PAL, NEA, NEO
PAL, NEA, NEO, GRL
Apostasioideae
Vanilloideae
Cypripedioideae
Epidendroideae
Orchidoideae
Asparagales MRCA
Orchidaceae MRCA
Orchids ancestral area
Givnish' ancestral area
(b)
90° N
90° S
90° W
45° W
0°
45° E
90° E
135° E
Paleo-Koeppen climatic zones
Marine
Dc/Db
Db
Da
Cc
Cb
Ca
Bwk
Bwh
Bsk
Bsh
As/Aw
Af/Am


(a)
No. spp
0
1000
2000
3000
4000
Ecuador
Colombia
New Guinea
Brazil
Peru
Venezuela
Borneo
Costa Rica
China
Mexico
Species richness
1
737
(b)
λ tip rates
0
0.5
1.0
1.5
2.0
0
200
Freq
1000
λ tip rates - mean
0.08
1.73
(c)
1 Talamancan PF
2 Costa Rican SMF
3 Napo MF
4 Cerrado
5 Guianan MF
λ tip rates - mean
0.092
1.19
Legend
Afrotropics
Australasia
Indomalaya
Nearctic
Neotropics
Paleotropics
No. records
λ tip rates
0
1
2
records
0
1000
2000
3000
4000
Ecoregion


Legend
Apostasioideae
Vanilloideae
Cypripedioideae
Epidendroideae
Orchidoideae
0.013
λ rate
2.1
(a)
K-Pg bounary
Origin of MTF
Diurideae
Cranichideae
Orchideae
IIX
VI
Neottieae
Sobralieae
Gastroideae
Tropidieae
Coelogyninae
X
Malaxideae
VII
Podochileae
Collabieae
IV
Epidendreae
II
V
I
Vandeae
Cymbidieae
III
IX
(b)
λ tip-rates
Lepanthes (I)
Pleurothallis (II)
Stelis (II)
Maxillaria (III)
Epidendrum (IV)
Pabstiella (II)
Pseudolepanthes (I)
Masdevallia (V)
Habenaria (VI)
Dracula (V)
Dendrobium (VII)
Anathallis (I)
Ponthieva (IIX)
Cranichis (IIX)
Tubella (I)
Lepanthopsis (I)
Encyclia (IV)
Trichosalpinx (I)
Caularthron (IV)
Platystele (II)
Scaphosepalum (II)
Oncidium (IX)
Specklinia (II)
Bulbophyllum (X)
No. spp.
Pleurothallis
Epidendrum
Masdevallia
Lepanthes
Habenaria
Maxillaria
Cypripedium
Vanilla
Dendrobium
Apostasia
λ tip rates
Species
Legend
Apostasioideae
Arethuseae
Collabieae
Cranichideae
Cymbidieae
Cypripedioideae
Diurideae
Epidendreae
Gastrodieae
Malaxideae
Neottieae
Nervilieae
Orchideae
Podochileae
Pogonieae
Sobralieae
Tropidieae
Vandeae
Vanilleae
Wullschlaegelideae
Xerorchideae
Outgroup
max λ tip rate
mean λ tip rate
min λ tip rate
Species number
80 Ma
60
40
20
0
CRETACEOUS - LATE
PALEO
EOCENE
OLIGO
MIOCENE
Pl
Q