Evolution of crassulacean acid metabolism (CAM) as an escape from ecological niche conservatism in Malagasy Bulbophyllum (Orchidaceae)

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Summary

- Despite growing evidence that niche shifts are more common in flowering plants than previously thought, little is known of whether such shifts are promoted by changes in photosynthetic pathways.
- Here we combine the most complete phylogeny for epiphytic Malagasy Bulbophyllum orchids (c. 210 spp.) with climatic niche and carbon isotope ratios to infer the group’s spatial-temporal history, and the role of strongly expressed crassulacean acid metabolism (CAM) in facilitating niche shifts and diversification.
- We find that most extant species still retain niche (Central Highland) and photosynthesis (C3) states as present in the single mid-Miocene (c. 12.70 million yr ago (Ma)) ancestor colonizing Madagascar. However, we also infer a major transition to CAM, linked to a late Miocene (c. 7.36 Ma) invasion of species from the sub-humid highland first into the island’s humid eastern coastal, and then into the seasonally dry ‘Northwest Sambirano’ rainforests, yet without significant effect on diversification rates.
- These findings indicate that CAM in tropical epiphytes may be selectively advantageous even in high rainfall habitats, rather than presenting a mere adaptation to dry environments or epiphytism per se. Overall, our study qualifies CAM as an evolutionary ‘gateway’ trait that considerably widened the spatial-ecological amplitude of Madagascar’s most species-rich orchid genus.

Key words: adaptive radiation, Bulbophyllum, crassulacean acid metabolism (CAM), diversification, Madagascar, niche evolution, Orchidaceae, phylogeny.

Introduction

Analyzing the extent to which species or clades are able to retain or shift their environmental niche space over time is key to understanding large-scale patterns of lineage diversification and adaptations to environmental change. The retention of niche-related ancestral ecological traits over time has been termed niche conservatism (Wiens et al., 2010), whereas evolutionary change that allows lineages to transverse niche boundaries is known as niche shift (Donoghue & Edwards, 2014). Niche conservatism has long been viewed as the dominant process in ecology across many angiosperm lineages (Crisp et al., 2004; Silvera et al., 2019; Li et al., 2019). Cam is rarely an all-or-nothing phenomenon as in CAM-exhibiting species the proportions of nocturnal CAM-type carbon gain vs diurnal C3 photosynthetic carbon gain can vary tremendously depending upon species and environmental conditions. Intraspecific variation often is associated with facultative CAM, which is the reversible induction or upregulation of CAM in response to water-deficit stress (Winter & Holtum, 2014). In plants with a strong CAM phenotype that gain carbon almost exclusively at night, overall transpirational water loss is reduced owing to stomatal closure for most of the day when the driving forces for water loss are high.
Stomata open predominantly at night concomitant with nocturnal uptake of CO₂ when the driving forces for water loss are low (Winter & Smith, 1996; Lüttge, 2004; Heyduk et al., 2019). CAM occurs in many succulent species of warm, semi-arid regions (e.g. Cactaceae), but also in many tropical vascular epiphytes that are subject to enormous short-term and seasonal variations in the availability of water (Keddy, 2007). CAM is well-known in tropical epiphytic species of Bromeliaceae and Orchidaceae, where CAM generally is considered a ‘key innovation’, promoting the invasion of new habitats and accelerating species diversification (Gravendeel et al., 2004; Motomura et al., 2008; Silvestro et al., 2013; Bouchenak-Khelladi et al., 2015; Givnish et al., 2015). However, strong evidence in support of this notion is hard to come by as ‘key innovations’ are difficult to test (e.g. Rabosky, 2014, 2017). For example, diversification rates were not significantly different between CAM and C₃ species of Afro-Malagasy Eulophiinae orchids (Bone et al., 2015). Moreover, in an Asian clade of the pantropical orchid genus Bulbophyllum Thouars, CAM species actually suffered from a 10-fold higher extinction rate compared to their C₃ con-generics (Hu, 2018). Thus, there is still a big gap in our knowledge regarding the extent to which CAM is linked to diversification and niche evolution in Orchidaceae, where less than 10% of all species (c. 26,000 in total; WCSP, 2020) have so far been surveyed for mode of photosynthesis (Silvera et al., 2009, 2010a,b; Winter, 2019; Torres-Morales et al., 2020).

Here, we study a monophyletic and mostly epiphytic (or rarely litho-/rheophytic) lineage of Bulbophyllum from Madagascar (c. 210 spp.; Sieder et al., 2009; Madagascar Catalogue, 2019) to further clarify the extent to which CAM, determined by carbon isotopic composition, is linked to niche evolution and species diversification in tropical orchids. We caution that the carbon isotope technique is not suitable to detect C₃-CAM intermediate species in which the CAM cycle, although present, does not contribute in a major way to total carbon gain relative to C₃ photosynthesis. However, it is an effective tool to identify species in which CAM is the principal mode of carbon assimilation (Winter et al., 2015). Based on a genus-wide phylogeny of nuclear ribosomal (nr) DNA (Gamisch & Comes, 2019), the radiation of Malagasy Bulbophyllum likely dates back to the mid-Miocene (c. 12.36 million yr ago (Ma)). Species richness of this lineage is highest in mid-elevation sub-humid forest (c. 800–1300 m above sea level (asl)) along the eastern slopes of the ‘Central Highlands’ (Cornet, 1974; Doder & Nowak, 2006; Fischer et al., 2007; Gamisch et al., 2016). However, members of a small subclade (termed ‘C’; sects. Bifalcula + Calamaria; c. 30 spp.; Gamisch et al., 2015, 2016) also occur in eastern-coastal, warm-humid evergreen rainforest and/or seasonally dry ’Northwest Sambirano’ rainforest (both c. 0–800 m asl; Gamisch et al., 2016), possibly reflecting major niche and/or physiological transitions during the history of Malagasy Bulbophyllum. However, in Bulbophyllum, a limited survey of carbon isotope ratios (δ¹³C/δ¹²C) has so far revealed only C₃ photosynthesis in Madagascar (Kluge et al., 1995), whereas Austral-Asian species are known to exhibit both C₅ and CAM-type isotopic signatures (Winter et al., 1983; Earnshaw et al., 1987; Hu, 2018).

Here, we report the most comprehensive (nuclear/plastid) phylogenetic and carbon isotope datasets for Malagasy Bulbophyllum to date, covering c. 85.2% and 90% of the total species diversity, respectively. Based on this, we then combine diversification rate analyses with ancestral climatic niche and photosynthetic pathway reconstructions to (1) infer the group’s spatial-temporal history; and (2) to test the role of strong CAM in facilitating niche shifts and diversification in Madagascar’s most species-rich orchid lineage. Moreover, we test hypotheses of broader biogeographical and evolutionary-physiological significance, namely that (3) insular radiations typically begin in marginal (coastal) rather than interior habitats (Liebherr & Hajek, 1990; Schluter, 2000); and that (4) CAM in tropical epiphytes may be selectively advantageous even in high rainfall forests (e.g. Pierce et al., 2002; Lüttge, 2004; reviewed in Martin, 2010), rather than presenting a mere adaptation to dry environments or epiphytism per se (e.g. Gravendeel et al., 2004; Silvera et al., 2009, 2010a,b; Bone et al., 2015).

Materials and Methods

Phylogenetic taxon sampling and molecular dating

We inferred phylogenetic relationships among 179 species of Malagasy Bulbophyllum (c. 85.2% of the group’s total (210 spp.) diversity; Sieder et al., 2009), using DNA sequence information from three nuclear (nrITS, PI, Xdh) and five plastid gene regions (atpH–atpH, ycf1, matK, trnD–trnE, psbA–trnH; see Supporting Information Table S1). This ingroup sampling includes 22 probably new but undescribed and five newly described species (Fischer et al., 2009; Hermans et al., 2021); as outgroups, we used nine species from Asia (four spp.), Africa (three) and the Neotropics (two) (Table S1). Sequences were obtained from GenBank (Fischer et al., 2007; Gamisch et al., 2015; Gamisch & Comes, 2019) or newly generated (Xdh, matK) following Gorniak et al., (2010) and Ford et al., (2009), aligned by CLUSTALW2 (Kearse et al., 2012) and edited manually. The total alignment of the 188 species (7712 bp) included 1504 sequences, c. 87% (1306) of which were complete (Table S1). Of the 7712 nucleotide sites, c. 15.4% (1191) were parsimony-informative (Table S2).

All plastid regions were combined and treated as a single partition, whereas the three nuclear regions were analyzed separately. For each partition, the best-fitting model of nucleotide substitution was identified (Table S2) using the size-corrected Akaike Information Criterion (AICc) in IQ-TREE v.1.6.2 (http://iqtree.cibiv.univie.ac.at; Trifinopoulos et al., 2016). Phylogenetic species relationships and divergence times were estimated in BEAST v.1.8.4 (Drummond et al., 2012) using age estimates of a previous genus-wide (nrITS) phylogeny (Gamisch & Comes, 2019) as secondary calibration points: (1) a uniform prior distribution ranging from 16.2 to 25.63 Ma at the Bulbophyllum root; (2) a normal prior of 12.26 ± 2.28 Ma (mean ± SD) for the crown of Malagasy Bulbophyllum; and (3) a normal prior of 5.67 ± 1.31 Ma for the crown of subclade C. A relaxed molecular clock with uncorrelated log-normal model was used (Drummond...
et al., 2006). The tree speciation prior followed a constant-rates birth-death (CR-BD) process, and one Markov chain Monte Carlo (MCMC) run was performed on the CIPRES Science Gateway portal (Miller et al., 2010) for $2 \times 10^8$ generations, sampling every 20,000th step. TRACER v.1.7.1 (Rambaut et al., 2018) was used to confirm that all parameters had sufficiently large effective sample sizes (ESS $> 233$) after discarding the first 10% of sampled trees as burn-in.

Assembly of georeferenced presence records and climatic niche datasets

In a first step, we assembled 5116 georeferenced presence records of Malagasy Bulbophyllum (coordinates available upon request), representing all named species plus 895 records of unknown identity. Records were sourced from own expeditions (2000–2018) in Madagascar (G.A.F., A.G. and Anton Sieder, Vienna) and La Réunion (A.G. and Ursula Jaros), the Global Biodiversity Information Facility (GBIF occurrence download: http://doi.org/10.15468/dr.ehvtif; 7 July 2017), and voucher specimens obtained from herbaria (i.e. Conservatoire et Jardin botaniques de la Ville de Genève (G), Royal Botanic Gardens Kew (K), Missouri Botanical Garden (MO), Université de Neuchâtel (NEU), Muséum National d’Histoire Naturelle (P), University of Salzburg (SZU), Parc Botanique et Zoologique de Tsimbazaza (TAN), Naturhistorisches Museum Wien (W), Universität Wien (WU)). Duplicate presence records per species and implausible locations (e.g. records with incorrect or swapped latitude and longitude, within ocean, capitals, urban areas, botanical gardens, etc.) were discarded. Slightly offshore coastal localities were snapped to the nearest land cell. In a further step, we used SPTHIN etc.) were discarded. Slightly offshore coastal localities were thus pruned from subsequent analyses.

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Identification of macroecological niche states

For identifying macroecological niche states, we first subjected dataset 1 (1206 locality-climate points) to principal components analysis (PCA) in R v.3.6.1. (R Core Team, 2019). To complement traditional PCA loadings, we also calculated $R^2$ values for each climate variable and ordinated axis (PC1, PC2) through regression of mean trait values per species on their respective mean PC scores to estimate which variables show highest loadings and correlations (i.e. were most important) (Folk et al., 2019). To investigate how these data points grouped in multidimensional climatic space, we used $k$-means clustering, as selected by CLVALID v.0.6-6 (Brock et al., 2011) when tested against alternative algorithms (e.g. hierarchical clustering, partitioning around medoids; results not shown). Accordingly, we used the $k$means function in NBCLUST v.3.0 (Charrad et al., 2014) to determine the optimal number of clusters ($K$) in this dataset based on 15 validity indices (kl, ch, hartigan, cindex, db, silhouette, ratkowsky, ball, ptbiserial, gap, frey, mcclain, dum, sindex, sdhub). Clustering and cluster visualization were carried out in CLUSTER v.2.0.6 (Maechler, 2017) and FACTOextra v.1.0.5 (Kassambara & Mund, 2017). This analysis identified three clusters (i.e. niches), hereafter referred to as ‘H’ (highland), ‘L’ (lowland) and ‘S’ (Sambirano; see Results).

Based on 731, 341 and 134 locality records assigned to niches $H$, $L$ and $S$, respectively (see Results), we generated ecological niche models (ENMs) for each niche separately, using MAXENT v.3.4.1 (Phillips et al., 2017) and only 10 (of 19) bioclimatic layers – thus, after removal of highly correlated variables (Pearson’s $r \geq 0.9$) in ENMTOOLS v.1.4.4 (Warren et al., 2010) to avoid potential overfitting. The retained variables were: annual mean temperature (bio1), isothermality (bio3), temperature seasonality (bio4), minimum temperature of coldest month (bio6), temperature annual range (bio7), annual precipitation (bio12), and precipitation, respectively, the wettest (bio16), driest (bio17), warmest (bio18) and coldest (bio19) quarters. Fifty bootstrap replicates and 75% of the localities were used to train the models. We averaged the area under the receiver operating characteristic (ROC) curve (AUC) across replicates to determine the predictive power of each model (AUC $> 0.7$; Fawcett, 2006). Suitable areas were defined as areas with higher occurrence (cloglog) probabilities than the ‘maximum training sensitivity plus specificity’ (MTSS) threshold (Liu et al., 2013). Niche range overlap was calculated with ENMTOOLS.

Reconstruction of ancestral niche states and estimation of transition rates

Ancestral niche states were reconstructed on the BEAST-derived maximum clade credibility (MCC) chronogram of Malagasy Bulbophyllum (with outgroups pruned before analysis) using the submodule MULTISTATE of BAYESTRAITS v.3.0 (Pagel et al., 2004). For each species, niche state ($H$, $L$ and/or $S$) was coded according to the mean ENM cluster suitability (above the MTSS) of its locality data (based on dataset 2). For instance, when the mean suitability of a given species was higher than the MTSS for $H$, then this species was coded as $H$. If, instead, a species had similar mean suitability above the MTSS for two or more states (i.e. owing to outliers and/or overlapping suitability in transitional zones between niches), a decision was made based on visual inspection of the species’ localities; otherwise, it was coded as ‘polymorphic’ (e.g. $HL$ or $LS$; see Table S3). Reversible-jump (rj) MCMC analyses were run for $5 \times 10^6$ generations, using a hyper-prior with an exponential prior (mean seeded from a uniform distribution}
on the interval 0 to 100), and a burn-in of \(5 \times 10^4\) generations. The reconstructed niche states were plotted for nodes of interest – the crown node of Malagasy *Bulbophyllum*, the crown nodes of major clades and all nodes of the ecologically diverse subclade C.

In addition, we used *BAYESTRAITS* to infer the direction of niche state transitions \(q\) by comparing a full (six-parameter) model with unconstrained parameters (i.e. \(q_{HL}, q_{LS}, q_{H1}, q_{L1}, q_{SL}, q_{SL}\)) to six models of zero unidirectional (i.e. asymmetrical) transitions for all pairwise combinations of states (i.e. \(q_{HL} = 0; q_{LS} = 0; \ldots q_{SL} = 0\)) (see Table S4). Model selection followed log\(_2\) Bayes Factors (BFs), with values \(> 6\) (or 10) considered as strong (or very strong) evidence for one model over the other (Kass & Raftery, 1995).

Reconstruction of ancestral photosynthetic pathways (C\(_3\) vs CAM)

Nocturnal fixation of atmospheric CO\(_2\) into malic acid is characteristic of CAM photosynthesis and results into a higher accumulation of \(^{13}\)C in plant carbon when compared to C\(_3\) photosynthesis (O’Leary, 1988; Bone et al., 2015). Thus, carbon isotope ratios \((^{13}\)C/\(^{12}\)C\)) can be used as proxy for photosynthetic pathway (Osmond et al., 1973). Accordingly, we performed leaf carbon isotope measurements in a total of 200 *Bulbophyllum* accessions, representing 191 different species (189 from Madagascar plus two outgroup species), 171 of which were included in the phylogeny of the present study (see also Table S3). Leaf samples were collected and silica-dried in the field (2000–2018) or obtained from herbarium specimens (WU, SZU, MO, P). About 2 mg per sample was subjected to carbon stable isotopic composition analyses (\(^{13}\)C) using the Thermo Scientific Delta V Advantage isotope ratio mass spectrometer (IRMS) at the Smithsonian Tropical Research Institute (STRI), Balboa/Panama City, Panama. \(^{13}\)C values were calculated relative to the Pee Dee Belemnite (PDB) standard (Belemnitella americana) using the formula: \(\delta^{13}\)C (‰) = \((^{13}\)C/\(^{12}\)C sample)/(\(^{13}\)C/\(^{12}\)C standard) – 1) \times 1000 (Silvera et al., 2009). Following Crain et al., (2004) and Winter et al., (2015), species with \(^{13}\)C values less or more negative than \(-20\)‰ were binary coded as strong CAM or C\(_3\), respectively (cf. Bone et al., 2015; Li et al., 2019; Winter, 2019). Although values less negative than \(-20\)‰ indicate CO\(_2\) fixation predominantly via CAM, values more negative than \(-20\)‰ do not preclude the possibility of low-level CAM, but instead indicate that this did not make a major contribution to carbon gain. Hence, unless stated otherwise in the text that follows, the term CAM always refers to strongly expressed CAM. Furthermore, it is safe to assume that in our study \(^{13}\)C values less negative than \(-20\)‰ are indicative of CAM and not of C\(_4\) photosynthesis because there is no direct evidence supporting the occurrence of C\(_4\) pathway (and associated ‘Kranz’ anatomy) in orchids (e.g. Hew & Yong, 2004).

Ancestral character states for C\(_3\) vs CAM were reconstructed in *BAYESTRAITS*, using the same methods and settings as described above for ancestral niche states. Furthermore, we performed separate runs in the *BAYESTRAITS* submodule *DISCRETE* to test for independent vs dependent (correlated) evolution of niche and photosynthetic states (Pagel & Meade, 2006). As this test requires binary traits, niche L was subsumed together with S, resulting in a niche coding H (highland) vs not H (or H vs LS). Model selection followed BFs.

Estimating rates of niche evolution and lineage diversification

For quantifying rates of niche evolution, we first subjected dataset 2 (3036 locality-climate points) to PCA (as described above) and calculated mean scores per species based on the first two components (PC1, PC2), respectively. We then used *BAMM* v.2.5.0 (Rabosky, 2014) to infer rates of niche evolution along the MCC chronogram (with outgroups pruned) by performing a run with \(10^9\) generations for each component (PC1, PC2) separately (BAMM, Bayesian Analysis of Macroevolutionary Mixtures). Likewise, we used *BAMM* to estimate rates of net lineage diversification \((r = \text{speciation} (\lambda) - \text{extinction} (\mu))\), taking incomplete taxon sampling into account (sampling frequency = 0.852). One rjMCMC run was performed with \(10^7\) generations, sampling every 1000th step under the default prior assumption of a single expected rate shift. Initial priors were obtained using the *setBAMMpriors* function in *BAMMTOOLS* v.2.1.6 (Rabosky et al., 2014). Post-analysis and visualization of rates through time were done in *BAMMTOOLS* after a 10% burn-in (see above).

In addition, we fitted twelve birth–death (BD) models to the MCC chronogram with either time-dependent (six models), palaeo-temperature-dependent (two) and palaeo-atmospheric-CO\(_2\)-dependent (two), or diversity-dependent (DD) diversification rates (two; see Table S5), using, respectively, the \(\text{fit\_bd}\) and \(\text{fit\_env}\) functions of *RPANDA* v.1.7 (Morlon et al., 2016) and the \(\text{dd\_ML}\) function of *DDD* v.3.7 (Etienne et al., 2012), with each analysis accounting for unsampled taxa (see above). Exponential diversification dependencies were specified (in \(\text{fit\_bd}\) and \(\text{fit\_env}\)) because linear diversification dependencies can lead to unrealistic model fits (Gamisch, 2020). The time curves of estimated mean global surface temperature and model inferred CO\(_2\) concentrations (Hansen et al., 2013) were smoothed in *RPANDA* using the default ‘NULL’ option for degrees of freedom (df; temperature = 100.06; CO\(_2\) = 207.79; note that the CO\(_2\) model is driven in part by the temperature model; hence, the two curves are not independent of each other; Hansen et al., 2013). The model with the lowest AIC\(_c\) score was considered the ‘best’, whereas those in which the difference to the best-fit model (AIC\(_c\)) was < 2 were regarded to have essentially equivalent fit (Burnham & Anderson, 2002). Note, all of the above analyses (*BAMM*; *RPANDA/DDD*) also were run separately on the ecologically diverse subclade C and the pruned tree without this lineage.

Finally, we used the *STRAPP* (Structured Rate Permutations on Phylogenies) procedure in *BAMMTOOLS* (Rabosky & Huang, 2016) to test for a significant relationship between mean \(r\) at the tips and the corresponding extent (1) quantitative niche states (PC1, PC2); or (2) the three macro-ecological niche states identified \((H, L, S;\text{ see Results})\). Statistical significance was calculated using Pearson’s correlation coefficient and the Kruskal–Wallis test, respectively, each based on 1000 permutations (two-tailed).
Results

Phylogeny reconstruction and divergence time estimates

Our BEAST-derived phylogeny based on three nuclear and five plastid gene regions (Figs 1, S1; Dataset S1) supported the monophyly of Malagasy Bulbophyllum (posterior probability, PP = 1.0) and identified three main clades (termed A, B and D), which essentially formed an unresolved trichotomy. Clades A (PP = 0.91) and B (PP = 1.0) harboured the great majority of species diversity (50 and 126 spp., respectively), whereas the three species of sect. Inversiflora (sensu Hermans et al., 2021) formed clade D (PP = 1.0). Notably, subclade C (sensu Gamisch et al., 2015, 2016), comprising sects. Bifalcata + Calamaria (30 spp.; PP = 1.0), occupied a nested and thus derived position within clade A, as likely sister of sect. Kainochilus (PP = 0.85; Fig. S1).

Based on our molecular dating, we estimated the mean crown age of Malagasy Bulbophyllum to the mid-Miocene (c. 12.70 Ma; 95% highest posterior density, HPD: 10.39–15.27 Ma). Clades A and B started to diversify shortly thereafter in quick succession at c. 11.58 (9.16–13.87) Ma and 10.92 (8.68–13.14) Ma, respectively. By contrast, subclade C began to radiate in the late Miocene, c. 7.34 (5.83–8.89) Ma. The great majority of extant species (123/179, c. 71.1%) likely originated during the Quaternary (c. 2.6 Ma; see Fig. S1).

Identification of macroecological niche states

For the 1206 locality-climate data of Malagasy Bulbophyllum (dataset I), five (of 15) k-means validity indices each suggested clustering of the samples into two or three groups, respectively (data not shown). However, we favoured K = 3 as the optimal (i.e. more informative) solution as this partition recovered essentially the same clusters (H, L, S) as previously inferred for subclade C (Gamisch et al., 2016). In the corresponding PCA biplot (Fig. 2), samples of clusters H (n = 731) and L (n = 341) differed mainly along PC1 (accounting for 55.9% of the total variance), which was strongly (R² > 0.7) and positively correlated with measures of temperature (bio1, 6, 8–11) and annual precipitation (bio12) (Table S6). By contrast, cluster S (n = 134) differed along PC2 (22%), which had relatively strong (R² > 0.4) and positive loadings of temperature seasonality (bio4) but negative ones of both isothermality (bio3) and precipitation seasonality (bio15) (Table S6). All pairwise comparisons between clusters revealed significant differences for each of the two axes tested (Wilcoxon–Mann–Whitney test, all P < 0.001).

The present-day ENMs generated for clusters H, L and S (hereafter ‘niches’) had high predictive power (AUC range: 0.926–0.958) and were largely parapatric in Madagascar (range overlap c. 0.06–0.12; Fig. 1a). When combined with the PCA results (Fig. 2; Table S6) and earlier phytogeographical (Humbert, 1955; Dransfield and Rakotoarivonjy, 2011) and climate data (Cornet, 1974; Yoder & Nowak, 2006; Gamisch et al., 2016), these niches can be characterized as follows: niche H extends from the eastern slopes to the plateau of the ‘Central Highlands’ (c. 800–1800 m asl) of sub-humid montane forest and sclerophyllous forest/scrubland, receiving relatively low annual rainfall and low annual (but variable seasonal) temperature; niche L largely coincides with the ‘Eastern Lowlands’ of coastal and humid rainforest (c. 0–800 m asl), featuring high annual rainfall at ‘intermediate’ annual and seasonal temperatures; and niche S occupies the seasonally dry Northwest ‘Sambirano’ rainforest (c. 0–800 m asl), maintaining high annual temperature and low annual (but variable seasonal) rainfall.

Ancestral niche states and transition rates

The Bayesian reconstruction of ancestral niche states (H, L, S) along the MCC chronogram (Fig. 1b) placed the crown node of Malagasy Bulbophyllum (c. 12.70 Ma) in the Central Highlands (H) with high probability (PP = 81.1), and the same was true for the crown nodes of clades B (PP = 87.2), D (PP = 78.1) and, to a lesser extent, A (PP = 57.5). In fact, most lineages and extant species of clades B and D retained this ancestral niche, with the exception of relatively few and independent transitions on terminal tip branches, mostly from H to the Eastern Lowlands (L) or, very rarely, from H to the Sambirano (S). Clade A, however, underwent a notable shift from H to L during the late Miocene, either coinciding with the origin of subclade C (stem node: PP = 50.6; c. 9.45 Ma) or, more likely, with its onset of diversification (crown node: PP = 80.8; c. 7.34 Ma); subsequently, relatively frequent transitions occurred (towards the tips) from L to S or H (or very rarely from S to L), resulting in a subclade with relatively high niche diversity (Fig. 1b). Nonetheless, across the entire phylogeny, ancestral niche conservatism (H) prevailed, with shifts occurring mainly from H to L and from L to S (Fig. 1b). In support of this, Bayestraits strongly rejected models opposing these two latter transitions (logBF qSL = −6.9; qLS = −10.4) but none with zero (unidirectional) transitions (i.e. qSH, qHL, qSL or qLS = 0; logBF = −1.0–0.9; see Table S4).

Reconstruction of ancestral photosynthetic pathways and correlation with niche states

Across the 200 Bulbophyllum accessions surveyed, δ13C values ranged from −37.75‰ to −12.09‰ (Table S3), resulting in a bimodal frequency distribution (Fig. 1c). Based on our binary classification (Winter et al., 2015), which codes the presence or absence of strongly expressed CAM, the great majority of the Malagasy species were found to exhibit C3 photosynthesis (159 of 190, c. 83.7%), whereas those engaging in CAM as major mode of carbon acquisition (31 of 190; c. 16.3%) were almost exclusively confined to (and fixed within) subclade C (29 of 31, c. 93.5%; Fig. 1b; Table S3). Accordingly, we identified C3 as the most likely ancestral state of Malagasy Bulbophyllum (crown node: PPc3 = 98.8), whereas the group’s major shift towards CAM coincided with the diversification of subclade C (crown node: PPCAM = 99.9). Otherwise, CAM evolved independently only twice, namely in B. petrae (sect. Polyradices; clade A) and B. cochinealloides (sect. Inversiflora, clade D). Using Bayestraits, we found strong support for correlated evolution (BF = 8.27) of C3 and CAM with, respectively, the ancestral Central Highland
Fig. 1 Ancestral climatic niche and photosynthetic pathway reconstructions of Malagasy Bulbophyllum orchids. (a) Current climate condition binary ecological niche models (ENMs) for macro-ecological niche clusters H, L and S of Malagasy Bulbophyllum species (based on dataset 1), using the MTSS threshold (inset shows La Réunion and Mauritius). (b) Reconstructions of ancestral character states along the BEAST-derived chronogram of Malagasy Bulbophyllum (see also Supporting Information Fig. S1) for photosynthesis pathways (purple: C₃; yellow: CAM; white: no data) and macro-ecological niches (green: cluster H (Central Highlands); black: L (Eastern Lowlands); orange: S (Northwest ‘Sambirano’)). Pie charts at selected nodes indicate posterior probability (PP) values. Capital letters (A–D) above nodes indicate major clades. See Fig. S1 for section abbreviations (coloured bars). The blue and red bottom curves indicate estimated global mean surface temperature (°C; Hansen et al., 2013) and model estimates of atmospheric CO₂ (ppm; Hansen et al., 2013), respectively. Ma, million yr ago. (c) Frequency histogram of carbon isotope ratio values (δ¹³C) of Malagasy Bulbophyllum (see also Table S3). Inset photograph shows habitus of the CAM species B. bicoloratum (sect. Calamaria). Photo credit: Rogier van Vugt.
niche \((H)\) and the derived Eastern Lowland/Sambirano niches combined \((LS)\).

Rates of niche evolution vs lineage diversification through time

For quantifying rates of niche evolution in BAMM, we used the mean PC1 and PC2 scores of each species based on the 3036 locality-climate dataset 2 (see Table S6 for component loadings). As for dataset 1, the primary loadings on PC1 vs PC2 captured measures of temperature/precipitation vs seasonality/isothermality. For the MCC chronogram with or without subclade C, niche evolution through time was consistently higher and more constant for PC1 relative to PC2 (Fig. 3a,b). Notably, for the full tree, niche evolution along PC2 started only in the late Miocene \((c. 8 \text{ Ma})\), almost coincident with the origin of subclade C \((c. 7.34 \text{ Ma})\), and then steadily increased toward the present. By contrast, in subclade C, niche evolution for PC1 and PC2 was temporally congruent and steadily increased toward the present at higher rates than in the remaining lineage (Fig. 3c).

Considering lineage diversification, we generally inferred relatively constant, yet slightly declining rates of net diversification \((\rho)\) throughout the history of Malagasy *Bulbophyllum*, regardless of the dataset used (i.e. full tree with or without subclade C, or the latter separately; Fig. 3a–c). Moreover, considering HPD intervals, none of the diversification rate parameters \((\rho; \text{speciation}, \lambda; \text{extinction}, \mu)\) differed significantly among the three datasets (Table S7).

According to the RPANDA/DDD results (Table S5), a pure-birth model of diversification (no extinction and constant speciation) best fitted the full tree, followed by a model that supported speciation as an exponential function of atmospheric CO\(_2\) \((\Delta \text{AICc} = 0.324)\); the reverse was true for the tree without subclade C included \((\Delta \text{AICc} \text{ (pure birth)} = 0.145)\). In both instances, however, models including palaeotemperature, CO\(_2\) or diversity-dependent (DD) speciation had essentially equivalent fit \((\sim 2 \Delta \text{AICc units})\), whereby the estimated carrying capacity \((K)\) was almost two to three times the current species richness \((K = 556/210 \text{ and } 400/171 \text{ spp., respectively})\). By contrast, for the CAM-possessing subclade C, a pure-birth model was decisive \((\Delta \text{AICc (second-best model)} = 2.273)\). Overall, these results provide no strong evidence for a potential link between diversification and global palaeoclimate change or density, suggesting instead that a pure-birth process might be sufficient to explain the diversification dynamics of Malagasy *Bulbophyllum* in general, and subclade C in particular.

According to STRAPP, there was no significant relationship between net diversification rate \((\rho)\) and niche state \((H, L, S)\) or multidimensional climate \((\text{PC1}, \text{PC2})\) space, whether estimated for the entire tree or subclade C (all \(P = 0.902–0.998)\). In sum, these results indicate that Malagasy *Bulbophyllum* diversified almost independently of a particular (qualitative or quantitative) niche state (see also Fig. 3).

Discussion

Spatial-temporal history of Malagasy *Bulbophyllum*

Based on a multilocus plastid/nuclear phylogeny, we dated the crown age of Malagasy *Bulbophyllum* to the mid-Miocene, \(c. 12.70 \text{ Ma} (95\% \text{ highest posterior density (HPD)}: 10.39–15.27 \text{ Ma})\) (Fig. 1). This timing is very similar to our previous estimate inferred from a single nuclear (nrITS) marker \((c. 12.36 (8.23–18.84) \text{ Ma); Gamisch & Comes, 2019)}\). Hence, the present results support our previous hypothesis that *Bulbophyllum* colonized Madagascar following the establishment of the Indian Summer monsoon \((c. 12.9–7.0 \text{ Ma})\) via a single long-distance dispersal event from the Asian-Pacific region \((Buerki et al., 2013; Gupta et al., 2015; Gamisch & Comes, 2019, and references therein)\). Concomitantly, our ancestral niche reconstructions (Fig. 1) show that the radiation of *Bulbophyllum* in Madagascar began in interior sub-humid environments of the ‘Central Highlands’ \((niche H)\), providing suitable and available (e.g. noncompetitive) ecological space required for successful island colonization \((Gillespie, 2007)}\). Because colonizers likely share similar niche conditions with their source populations \((Wiens & Graham, 2005; Wiens et al., 2010; Steinbauer et al., 2016)\), the ancestor of Malagasy *Bulbophyllum* probably originated from similar habitats in the Asian-Pacific region (e.g. mountains of the Sunda Islands, New Guinea Highlands, India, Indochina and South China). In turn, these results dismiss the hypothesis that insular radiations typically begin in marginal (coastal) habitats
fringing the island (Liebherr & Hajek, 1990; Schluter, 2000), as recently shown for Malagasy Canarium (Buseraceae; Federman et al., 2015).

Major transition from C3 to CAM, associated with invasion of high rainfall forest

We observed a strong bimodal frequency distribution of δ13C values in Malagasy Bulbophyllum (Fig. 1c; Table S3). This is consistent with previously published large-scale species surveys of both Orchidaceae (Silvera et al., 2010b; Torres-Morales et al., 2020) and Bromeliaceae (Crayn et al., 2015) likewise showing a frequency minimum of about −20 ‰, a cluster of δ13C values more negative than −20 ‰, composed largely of C3 species, and a cluster of δ13C values less negative than −20 ‰, principally composed of species that engage in CAM as the dominant mode of carbon assimilation. However, for each family, species of the C3 cluster also are known to exhibit some degree of CAM activity, based on measurements of nocturnal increases in titratable acidity and nocturnal net CO2 uptake (for Orchidaceae, see Silvera et al., 2014). Such species have been defined as ‘C3-CAM intermediates’ (Winter et al., 2015). In our study, photosynthetic pathway classification is based on δ13C values alone, and does not include physiological measurements on living plants. Therefore, rather than considering any degree of CAM activity relative to C3, our approach is focused exclusively on the transition of Bulbophyllum to strongly expressed CAM, and thus on species in which the adaptive significance of CAM is expected to matter most.

Our results clearly identify C3 as the ancestral and predominant mode of photosynthesis in Malagasy Bulbophyllum (159 of 190 spp., c. 83.7%), whereas strong CAM is relatively rare (31 of 190, c. 16.3%) and mostly confined to subclade C (29 of 31, c. 93.5%), except for two independent, species-specific transitions (B. petrae, B. cochlitoaloides; Fig. 1b; Table S3). Thus, the majority of species still occupying the ancestral ‘Central Highlands’ niche (H) exhibit C3 photosynthesis, whereas CAM is mostly associated with relatively rare niche shifts to either the ‘Eastern Lowlands’ (L) or the ‘Sambirano’ (S), as found mostly in subclade C (Fig. 1b). Hence, Malagasy Bulbophyllum features considerable ecological niche (H) and physiological (C3) conservatism, yet these phylogenetic constraints (sensu Wiens et al., 2010) were apparently not strong enough to restrain the correlated evolution of alternative (derived) niche states (L, S) and CAM. More specifically, the group’s major transition from C3 to CAM most likely occurred at the crown node of subclade C (PP = 99.9), i.e. during the late Miocene (c. 7.36 Ma; 95% HPD: 5.83–8.89 Ma), and was accompanied by a major niche shift from the sub-humid Central Highlands (H) to the warmer and moister Eastern Lowlands (L), followed by colonizations of the hotter/seasonally dry ‘Sambirano’ (S) or reversals back into the highlands (Fig. 1b; Table S4).

Based on these ancestral niche reconstructions, it appears unlikely that CAM evolved initially in Malagasy Bulbophyllum as an adaptive response to dry environments or epiphytism per se (e.g. Gravendeel et al., 2004; Silvera et al., 2009, 2010a,b; Bone et al., 2015). Instead, our data suggest that CAM initially served as a ‘gateway’ trait (sensu Donoghue & Edwards, 2014; Bouchenak-Khelladi et al., 2015), facilitating the invasion of a novel adaptive zone of high rainfall coastal forest (L). Even though puzzling at first glance, CAM in wet and/or humid environments also is known from other (sub) tropical epiphytes (e.g. Bromeliaceae: Aechmea; Pierce et al., 2002; pteridophytes: Silvera et al., 2010a; and references therein). Although still poorly understood, numerous eco-physiological and adaptive hypotheses have been advanced to explain this “apparent conundrum”, also in relation to the C4 pathway (reviewed in Martin, 2010). The best-investigated and perhaps most plausible explanations of CAM

Fig. 3 Rates-through-time plots, derived from Bayesian Analysis of Macroevolutionary Mixtures (BAMM), for mean rates of net diversification (r) and climatic niche evolution (PC1, Principal component 1; PC2, Principal component 2) in Malagasy Bulbophyllum. Plots are shown for (a) the full tree of this orchid lineage (with outgroups excluded; see Supporting Information Fig. S1); (b) the tree without subclade C; and (c) subclade C taken separately. The shaded areas indicate the 95% confidence interval. The time curves of estimated mean global surface temperature (°C) and model estimates of atmospheric CO2 (ppm) data are derived from Hansen et al. (2013) (see also Fig. 1b). Ma, million yr ago.
under high rainfall conditions include: (1) the stimulation of CO₂ uptake following the wetting of leaves (stomata blockage) when subsequently dry (Pierce et al., 2002); (2) the ability to assimilate CO₂ over extended time periods (i.e. not only during night but also in early/late daytime; Lüttge, 2004; see also Keeley, 1998, for CAM in aquatic plants); or (3) water conservation when rainfall periods are too short and/or infrequent (Martin, 2010). Hence, in Malagasy Bulbophyllum, CAM probably not only proved advantageous for species invading the wetter Eastern Lowlands (L), but also provided a pre-adaptation to the seasonally dry Northwest ‘Sambirano’ rainforest (S). Overall, this shift in photosynthetic pathway significantly widened the spatial-ecological amplitude of the entire group, and might also have favoured the frequent niche transitions observed within subclade C (Fig. 1b; Table S4; see also Gamisch et al., 2016).

But what extrinsic (e.g. climatic) factor could have caused, or at least promoted this major shift towards CAM? Rapid aridification under low CO₂ conditions in the mid-to-late Miocene frequently is cited as potential factor promoting the evolution of CAM (e.g. Bone et al., 2015; Hu, 2018; Li et al., 2019). However, we found no strong (unambiguous) evidence for such a potential link between inferred historical changes in CO₂ (or temperature) and diversification across the full tree, and not at all for subclade C (Table S5). However, the crown age of this subclade and its corresponding shifts towards CAM and into the Eastern Lowlands (c. 7.36 Ma) remarkably coincide with the strengthening of the Indian Summer monsoon (c. 7 Ma; Gupta et al., 2015), resulting in increased moisture levels throughout eastern Madagascar and, possibly, the establishment of the Northwest ‘Sambirano’ (Yoder & Nowak, 2006; Buergi et al., 2013; Andriananjamanantsoa et al., 2016). In addition, these changes in monsoon climate could have created environmental instability across the comparatively older sub-humid and humid forest biomes of the Central Highlands and Eastern Coast of Madagascar, respectively (Cornet, 1974; Buergi et al., 2013). It is tempting to speculate that these climatic vicissitudes during the late Miocene especially favoured Bulbophyllum species with ‘obligate, but flexible CAM’ (sensu Lüttge, 2004; e.g. capable of daytime CO₂ uptake) to move from sub-humid highland to wetter or seasonally dry lowland niches. Nonetheless, it is worth noting that CAM in its most plastic form – facultative CAM induced or upregulated by environmental (e.g. climatic) factors – has not yet been demonstrated unequivocally for any species of the Orchidaceae (Winter & Holtsm, 2014).

Rates of species diversification and niche evolution in relation to CAM

According to adaptive radiation theory (Simpson, 1944; Schluter, 2000), lineages exposed to ecological opportunity, and especially after island colonizations (Gillespie, 2007), are expected to show an ‘early-burst’ of both species diversification and phenotypic evolution, followed by a slowdown in these rates as a consequence of limits imposed by geographical area, species competition and/or niche availability (Harmon et al., 2008; Rabosky, 2009; Silvestro et al., 2013; Givnish et al., 2014; Herrera, 2017; Linder & Bouchenak-Khelladi, 2017; Burbrink et al., 2019). In Madagascar, such patterns of declining diversification rates have been reported in Angraecum orchids (Andriananjamanantsoa et al., 2016), and several amphibian/reptile and bird lineages (e.g. Jönsson et al., 2012; Reddy et al., 2012; Scantlebury, 2013).

However, our rate-through-time analyses (Fig. 3) indicate (1) mostly constant rates of speciation with little or no extinction in Malagasy Bulbophyllum (with full tree mean μ = 0.06), including no strong evidence for density dependence (Table S5); and (2) elevated rates of niche (PC2) evolution within the last c. 8 million yr, largely reflecting the widened climatic niche space of the CAM-possessing subclade C (in terms of seasonality/isothermality; Table S6). Together, these results indicate that limits for both species diversity and niche evolution have not yet been reached in this orchid radiation (Rabosky, 2009), despite its relative antiquity (late Miocene) and insular setting. We suspect the lack of spatial-ecological boundaries in Malagasy Bulbophyllum could be caused by a combination of factors, including general features of tropical orchids (e.g. low population sizes: McCormick & Jacquemyn, 2014; Hu et al., 2017; vast surface area of their mostly epiphytic life zone: Gravendeel et al., 2004), lineage-specific characteristics (e.g. relative unspecialized pollination by a single functional group of fly pollinators: Fischer et al., 2007; Gamisch et al., 2014) and, perhaps foremost, historical contingencies (e.g. Moen & Morlon, 2014), especially the evolution of CAM, providing a mechanism to escape from the ancestrally highland niche (H) into a wider range of lowland (L, S) niches (see Discussion above).

However, despite these continuing ecological opportunities, we found no significant differences in diversification parameter estimates (rates of speciation, λ, extinction, μ, and net diversification, ρ) between the CAM-possessing subclade C and the full tree with or without this lineage (Table S7). This result contrasts with those from previous studies demonstrating a clear link between the origin of CAM and increased r in both Bromeliaceae (Silvestro et al., 2013; Bouchenak-Khelladi et al., 2015; but see Givnish et al., 2014) and Euphorbiaceae (Horn et al., 2014); however, no such association has so far been reported for Orchidaceae (Afro-Malagasy Eulophiinae: Bone et al., 2015; Asian Bulbophyllum: Hu, 2018; this study). Hence, collectively, these latter studies challenge the idea of CAM as a ‘key innovation’ in tropical orchids (e.g. Gravendeel et al., 2004; Givnish et al., 2015). However, we are not aware of similar studies reporting not only near constant diversification rates in a large endemic Malagasy plant lineage, but also a major CAM-mediated niche shift from comparatively drier to moister habitats (here from niches H to L), whether in this island or elsewhere.

Conclusions

The likely single species of Bulbophyllum colonizing Madagascar in the mid-Miocene (c. 12.70 Ma) arrived in the Central High-land region and exhibited C₃ photosynthesis. These niche and metabolic states are retained by the great majority of extant species. Nonetheless, a major shift from C₃ to CAM, coinciding with the crown age of subclade C (c. 7.36 Ma), provided a
mechanism to escape from the ancestrally conserved sub-humid Central Highland niche, and to first move into the Eastern Lowland rainforests, and then into the seasonal dry Northwest “Sam-birano”. Hence, CAM in these mostly epiphytic orchids is best characterized as a ‘gateway’ trait, first conveying a selective advantage in coastal forest habitats with high rainfall (e.g. possibly compensating for wetted leavesblocked stomata), and then pre-adapting such lowland species to further colonize seasonally dry habitats. Although the shift towards CAM resulted in elevated rates of climatic niche evolution, it had no effect on species diversification. Nevertheless, evolutionary change in this single physiological trait had a major role in shaping the evolution of Madagascar’s most species-rich orchid genus by increasing its overall spatial-ecological amplitude and ‘total diversification’, namely species richness (see also Rabosky, 2017).

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Author contributions

AG and HPC conceived and designed the study; AG performed the analyses; AG analyzed the data; KW supervised the IRMS analyses; GP contributed sequences; AG, HPC and KW interpreted the data; and AG and HPC drafted the manuscript with contributions by KW. All authors read and approved the final manuscript.

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Data availability

The raw phylogenetic tree associated with Figs 1 and S1 is available as Dataset S1. The underlying sequence data generated for this study were deposited at GenBank (see Table S1). All other relevant intermediate data are available from the authors upon request.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Dataset S1** BEAST-derived MCC phylogenetic tree in nexus file format.

**Fig. S1** BEAST-derived MCC chronogram of Malagasy *Bulbophyllum*, plus outgroups.

**Table S1** DNA sequence information for 179 species of Malagasy *Bulbophyllum* and nine outgroup taxa used for phylogenetic inference (see Figs 1b, S2).

**Table S2** Features of the four DNA partitions used for phylogenetic inference of Malagasy *Bulbophyllum*.

**Table S3** List of *Bulbophyllum* species analysed for photosynthetic pathway (CAM vs C3) and macroecological niche states.

**Table S4** Testing alternative models of niche transition in Malagasy *Bulbophyllum*.

**Table S5** Time, palaeo-environmental, and diversity-dependent diversification models.

**Table S6** Loadings and $R^2$ values of bioclim variables (bio1–19).

**Table S7** BAMM-derived diversification rate estimates.

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