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Nuclear–plastid discordance indicates past introgression in *Epidendrum* species (Laeliinae: Orchidaceae) with highly variable chromosome numbers

EDLLEY M. PESSOA[1,2,*], FELIPE NOLLET[3], RAFAEL F. MAGALHÃES[4], JUAN VIRUEL[5], FÁBIO PINHEIRO[6] and MARK W. CHASE[5,7]

[1]*Laboratório de Estudos Integrados de Plantas, Departamento de Botânica e Ecologia, Universidade Federal de Mato Grosso, Cuiabá, Mato Grosso, Brazil*
[2]*Programa de Pós-Graduação em Biodiversidade, Ambiente e Saúde, Universidade Estadual do Maranhão, Caxias, Maranhão, Brazil*
[3]*Departamento de Biologia, Laboratório de Sistemática e florística de Angiospermas, Universidade Federal Rural de Pernambuco, Recife, Pernambuco, Brazil*
[4]*Departamento de Ciências Naturais, Universidade Federal de São João del-Rei, São João del-Rei, Minas Gerais, Brazil*
[5]*Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3DS, UK*
[6]*Departamento de Biologia Vegetal, Universidade Estadual de Campinas, São Paulo, Brazil*
[7]*Department of Environment and Agriculture, Curtin University, Perth, Western Australia, Australia*

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Here we study a challenging group of karyotypically highly variable *Epidendrum* spp. using phylogenetic methods to help understand how hybridization/introgression contributes to karyotype evolution. We hypothesize that species with great chromosome number variation are a result of past hybridization/introgression. Conflicting topologies in trees constructed using separate plastid and nuclear datasets suggest past hybridization events that occurred most probably at least 3.7 Mya. A basic number $x = 14$ and substantial karyotype change followed by species divergence are suggested. Descending dysploidy and polyploidy were the most frequent changes estimated across the phylogenetic tree of the group. Two species, *Epidendrum secundum* and *E. xanthinum*, have probably experienced unidirectional gene flow involving their ancestors (the pollen recipients) and ancestors of *E. puniceoluteum* and *E. denticulatum* / *E. flammeum*, respectively, the pollen donors. However, it is not possible to say whether hybridization participated in the origin of *E. secundum* and *E. xanthinum* or merely contributed to their genomic divergence and karyotype change through introgression as has been observed in modern hybrid zones in *Epidendrum*. This pattern of introgression causing karyotype disruption and divergence could help explain the enigma of some highly diverse genera, such as *Epidendrum*. Further studies using a wider sampling of the genus could test if gene flow and karyotype variability are associated with the increase of speciation rates.

ADDITIONAL KEYWORDS: cytogenetics – Epidendroideae – evolution – past hybridization events – phylogenetic incongruence.

INTRODUCTION

In addition to observation of intermediate morphological features between putative parental species (Rieseberg & Ellstrand, 1993), asymmetrical bimodal karyotypes and chromosome number variation in some species may serve as a source of evidence of past hybridization events (Yamagishi-Costa, 2009; Mckain *et al.*, 2012; Weiss-Schneeweiss & Schneeweiss, 2013; Medeiros-Neto *et al.*, 2017; Garcia *et al.*, 2018). Furthermore, phylogenetic incongruence in or between different genomes (nuclear/mitochondrial/plastid) is another source of evidence to infer putative ancient hybridization and introgression (Rieseberg & Soltis, 1991;

*Corresponding author. E-mail: edlley_max@hotmail.com

Wendel & Doyle, 1998; Sang & Zhang, 1999). However, incongruent phylogenetic patterns can be the result of events other than hybridization, such as technical issues (misidentification, contamination, uncertainty in phylogenetic reconstruction or inaccurate orthology assessment), analytical artefacts (e.g. branch attraction; Guo, Thomas & Saunders, 2018), horizontal gene transfer (HGT; Davis & Xi, 2015) and incomplete lineage sorting (ILS; Wendel & Doyle, 1998; Goldman *et al.*, 2004; Pelser *et al.*, 2010; Som, 2015; Dodsworth *et al.*, 2020). Because HGT is rare among autotrophic angiosperms, ILS and hybridization/introgression emerge as the two main causes of phylogenetic incongruence in the absence of technical and analytical issues (Guo *et al.*, 2018). Distinguishing between these two processes is challenging, but recent coalescent approaches have been developed to reconstruct species trees while accounting for ILS (Maureira-Butler *et al.*, 2008; Liu, Yu & Edwards, 2010; Pelser *et al.*, 2010; Blanco-Pastor, Vargas & Pfeil, 2012; Chifman & Kubatko, 2014; Mirarab *et al.*, 2014; Guo *et al.*, 2018).

Phylogenetic incongruence between nuclear and plastid-based trees has been used to infer ancient hybridization/introgression in various groups of angiosperms (e.g. Dodsworth *et al.*, 2020; Dong *et al.*, 2021; Rose *et al.*, 2021), including Orchidaceae (Felix & Guerra, 2005; Koehler *et al.*, 2008; Van den Berg *et al.*, 2009; Oliveira *et al.*, 2015; Pérez-Escobar, Balbuena & Gottschling, 2016). Orchidaceae are one of the most diverse plant families in terms of the number of species (Christenhusz & Bing, 2016), and they include some species-rich genera such as Neotropical *Epidendrum* L. with > 1500 species (Hágsater & Soto-Arenas, 2005). *Epidendrum* has been a focus for evolutionary studies (Pinheiro & Cozzolino, 2013; Pessoa *et al.*, 2021), and recent phylogenetic studies have suggested the existence of discordant phylogenetic topologies between nuclear ribosomal and plastid datasets (Klein *et al.*, 2019; Mendoza *et al.*, 2020; Pessoa *et al.*, 2021). Both these commonly used marker categories evolve in atypical ways compared to most nuclear loci: plastid DNA is predominantly non-recombining and uniparentally (usually maternally) inherited, whereas nuclear ribosomal (nr) DNA is initially biparentally inherited but before recombination can advance far one parental type soon predominates and the other is eliminated by gene conversion/concerted evolution (Baldwin, 1992; Chase *et al.*, 2003). These traits make interpretation of incongruent phylogenetic trees constructed from plastid and nrDNA difficult because they can be the result of species having a hybrid origin or, conversely, rare introgression that combines with a subsequent bottleneck to produce marker capture (Eidesen, Alsos & Brochmann, 2015; Schutz *et al.*, 2016). Although we recognize their limitations, these two types of phylogenetic makers are suitable for addressing questions relating to the factors involved in the origin of the karyotypic diversity in some *Epidendrum* spp.

Polyploidy has been widely recorded for *Epidendrum* (Tanaka & Kamemoto, 1984; Felix & Guerra, 2010; Assis *et al.*, 2013), especially in species related to *E. secundum* Jacq., *E. nocturnum* L. and *E. smaragdinum* Lindl. (informal species groups *sensu* Hagsáter, 1985) that exhibit variation in chromosome number and morphology (Pinheiro *et al.*, 2009; Felix & Guerra, 2010; Assis *et al.*, 2013). Although the most probable basic number of the genus is $x = 10$, $2n = 40$ is the most common chromosome number in *Epidendrum* (Assis *et al.*, 2013) and in all genera of Laeliinae (Felix & Guerra, 2010; Nollet *et al.*, 2022). The main hypothesis is that *Epidendrum* has arisen from an ancestor with $2n = 20$ that underwent polyploidy during its early diversification. Recent studies have shown large variation in chromosome numbers among Brazilian species in the *E. secundum* group (informal group of species *sensu* Hagsáter 1985), often due to polyploidy followed by events of ascending and/or descending dysploidy (e.g. $2n = 24, 28, 30, 40, 42, 48, 50, 52, 54, 56, 58, 60, 68, 70, 72, 80, 84, 120, 220, 224, 240$) and asymmetrical and/or bimodal karyotypes (Tanaka & Kamemoto, 1984; Pinheiro *et al.*, 2009; Felix & Guerra, 2010; Assis *et al.*, 2013). In this context, Nollet *et al.* (2022) suggested that the group has an allopolyploid origin followed by dysploidy with $x_2 = 14$. Indeed, several hybrid zones have been reported between species related to *E. secundum* group. In most cases, parental species show extensive differences in chromosome number, producing sterile (Pinheiro *et al.*, 2015) to mostly sterile hybrids experiencing subsequent introgression despite large differences in chromosome numbers (Pinheiro *et al.*, 2016; Arida *et al.*, 2021). This introgression is made possible by their longevity and capacity for vegetative reproduction, allowing rare successful reproduction to be detected.

Cardoso-Gustavson *et al.* (2018) estimated divergence times on a phylogenetic tree of *Epidendrum*. These authors reconstructed a relatively young age in the late Miocene (12 Mya) for the crown node of the genus, and the split of the clade of Brazilian species related to *E. secundum* was dated to 4.37 Mya. Re-analysing the molecular datasets used in that study, we noted strongly supported discordance between plastid and nrITS-based trees for a group of species included in this Brazilian clade. In this study, we aim to investigate the factors underpinning these incongruent tree topologies and their link with chromosome number variability. Here, we hypothesize that past hybridization/

introgression events are involved in creation of substantial chromosome number variation in several *Epidendrum* spp. independent of polyploidy.

MATERIAL AND METHODS

STUDY GROUP

Epidendrum as currently circumscribed (Hágsater & Soto-Arenas, 2005) lacks a formal infrageneric classification based on phylogenetic reconstruction. The proposals of Cogniaux (1898–1902), Pabst & Dungs (1975) and Brieger (1976–1977) share most features with the original classification by Lindley (1841, updated in 1853). One of Lindley's subgenera that is easily recognized by possession of erect cylindrical stems with several distichous coriaceous leaves and long-peduncled inflorescences covered with closely spaced sheaths is *E.* subgenus *Amphiglottium* Lindl. (Lindley 1841, 1853). However, recent phylogenetic studies resolved the larger circumscription as non-monophyletic (Hágsater & Soto-Arenas, 2005; Klein *et al.*, 2019). Five sections were proposed by Lindley (1853) in this subgenus: *Polycladia* Lindl., *Holochila* Lindl., *Schistochila-Integra* Lindl., *Schistochila-Carinata* Lindl. and *Schistochila-Tuberculata* Lindl. The last three were combined by Brieger (1976–1977) in *E.* section *Amphiglottium* (Lindl.) Brieger and subsequently reorganized as three subsections: *Integra* (Lindl.) Brieger, *Carinata* (Lindl.) Brieger and *Tuberculata* (Lindl.) Brieger.

More recently, Hágsater (1985) organized *Epidendrum* in informal species groups that have been widely applied in recent systematic studies (e.g. Carnevali & Romero, 1992; Pessoa *et al.*, 2012, 2021; Pessoa, Felix & Alves, 2014; Barberena & Gonzaga, 2016; Pessoa, Miranda & Alves, 2016; Klein *et al.*, 2019). The species of *E.* section *Amphiglottium sensu* Brieger (1976–1977) were mostly included in the following three species groups: *E. anceps* Jacq., *E. secundum* and *E. smaragdinum*. The phylogenetic relationships among Brazilian species of *E.* section *Amphiglottium*, mostly members of the *E. secundum* group (*sensu* Hágsater, 1985), were studied by Pinheiro *et al.* (2009), who found them to be monophyletic. The proposals of Lindley (1853) and Brieger (1976–1977) to split *E.* subgenus *Amphiglottium* into three sections/subsections (*Integra*, *Carinata* and *Tuberculata*) were not supported. Pinheiro *et al.* (2009) also found that the species previously grouped in the section/subsection *Carinata*, characterized by two globose calli at the base of the lip blade and a keel projecting longitudinally over the midlobe, were better reorganized based on their distributions, which they named the Atlantic and Andean–Guyanan clades. The Atlantic clade comprises five species endemic to eastern Brazil (Pessoa, 2020): *E. cinnabarinum* Salzm. ex Lindl., *E. denticulatum* Barb.Rodr., *E. flammeum* E.Pessoa & M.Alves, *E. fulgens* Brongn. and *E. puniceoluteum* F.Pinheiro & F.Barros (all section/subsection *Carinata*; Fig. 1).

Hybrid zones have been reported for some of these species, and introgression with *E. secundum* and *E. xanthinum* Lindl. (both section/subsection *Tuberculata*) has been identified (Pinheiro *et al.*, 2010, 2015; Nollet *et al.*, 2022). Six hybrid zones are known among these species: *E. fulgens* × *E. puniceoluteum* (Moraes *et al.*, 2013), *E. fulgens* × *E. denticulatum* (Pinheiro *et al.*, 2015), *E. secundum* × *E. xanthinum* (Pinheiro *et al.*, 2016; Nollet *et al.*, 2022), *E. secundum* × *E. ibaguense*, *E. secundum* × *E. flammeum* (Assis *et al.*, 2013; Nollet *et al.*, 2022) and *E. denticulatum* × *E. orchidiflorum* (Arida *et al.*, 2021). In the Andean region, hybrid swarms involving more than two species have also been found (Vega *et al.*, 2013). Study of these has established basic chromosome numbers from 'pure' populations and others that are clearly products of recent polyploidy, hybridization or introgression (Table 1).

Great variation in chromosome numbers has been described in the species of the Atlantic clade, in the range $2n$ = 24–240 and characterized morphologically by asymmetrical bimodal karyotypes (Tanaka & Kamemoto, 1984; Guerra, 2000; Pinheiro *et al.*, 2009, 2015; Felix & Guerra, 2010; Assis *et al.*, 2013; Moraes *et al.*, 2013; ; Nobrega *et al.*, 2017; Cordeiro, 2019; Nollet *et al.*, 2022). *Epidendrum secundum* and *E. xanthinum* are even more variable (Table 1), and intraspecific polyploids and several cytotypes have been reported within and between populations (Assis *et al.*, 2013).

PHYLOGENETIC ANALYSES

The five species of the Atlantic clade, two representatives of the Andean–Guyanan clade (*E. ibaguense* and *E. macrocarpum*), and *E. secundum* and *E. xanthinum* (section/subsection *Tuberculata*) were included in the analyses. *Epidendrum flexuosum* G.Mey was chosen as an outgroup following Cardoso-Gustavson *et al.* (2018). We used sequences of three plastid spacers (*rpl32-trnL*, *trnL-trnF* and *trnT-trnL*), the plastid gene *matK* and the nuclear internal transcribed spacer (nrITS) available in GenBank (Pinheiro *et al.*, 2009; Pessoa *et al.*, 2012; Vieira *et al.*, 2017; Cardoso-Gustavson *et al.*, 2018).

One representative per species was included in both plastid and nrITS analyses (ten samples; Supporting Information, Appendix S1), and for the latter a new phylogenetic reconstruction was performed using multiple samples of *E. secundum* and *E. xanthinum* from Brazil and other countries (30 samples; Appendix S2). We newly sequenced five ITS sequences of *E. secundum* and ten of *E. xanthinum* (Appendix

Figure 1. Species of the Atlantic clade. A, *Epidendrum cinnabarinum*; B, *Epidendrum denticulatum*; C, *Epidendrum flammeum*; D, *Epidendrum fulgens*; E, *Epidendrum puniceoluteum*.

S2, including vouchers) following the procedures described by Cardoso-Gustavson (2018). The second analysis was performed to determine if the position of these two species was not due to a technical issue (misidentification, contamination or uncertainty in phylogenetic reconstruction) and to determine if there was nrITS allelic variation among accessions of these species that could be traced to different alleles being retained.

Alignments were generated using MUSCLE (Edgar, 2004) as implemented in the Geneious platform (Biomatters, Auckland, New Zealand). Bayesian inference (BI), maximum-likelihood (ML) and maximum-parsimony (MP) analyses were performed for each matrix using, respectively, MrBayes 3.2 (Ronquist *et al.*, 2012), RAxML 8.1.20 (Stamatakis, 2014) and PAUP 4.0b10 (Swofford, 2002). BI and ML were run in the CIPRES Science Gateway portal (Miller *et al.*, 2010). The best-fitting nucleotide substitution model for each dataset was selected using JModelTest 2.1.5 (Dariba *et al.*, 2012) under the Akaike information criterion (AIC). The most appropriate model was GTR for the plastid markers and GTR+G for nrITS.

BI analysis was performed with two independent simultaneous runs and four chains each, the Markov chain Monte Carlo (MCMC) parameters were set to four million generations, sampling every 400 trees, and we discarded as burn-in the first 2500 trees (25%). Convergence between the two independent runs was checked with Tracer 1.6 (Rambaut *et al.*, 2018) using the estimated sample size value (> 200). ML was performed with 1000 pseudoreplicates of thorough bootstrapping, and MP analyses were performed via heuristic searches with 1000 random taxon-addition pseudoreplicates and tree-bisection-reconnection (TBR) branch swapping. Bootstrap percentages (BP) were estimated with 1000 non-parametric pseudoreplicates and TBR swapping. Phylogenetic trees were edited using FigTree v.1.3 (Rambaut, 2018).

Although long-branch attraction (Felsenstein, 1978) is not expected for such a young group of species, its presence was visually examined by analysing

Table 1. Chromosome numbers of the analysed species retrieved from Tanaka & Kamemoto (1984)[1], Guerra (2000)[2], Pinheiro *et al.* (2009)[3], Felix & Guerra (2010)[4], Assis *et al.* (2013)[5], Moraes *et al.* (2013)[6], Pinheiro *et al.* (2015)[7], Nobrega *et al.* (2017)[8], Nollet *et al.* (2022)[9] and Cordeiro (2019)[10]. Bold: putative 'pure' populations

	Chromosome numbers
E. secundum	**28**, 30, 40, 42, 48, 50, 52, 54, **56**, 58, 60, 68, 80, **84**[3,5]
E. xanthinum	**28**, 30, 40, 60, 80[3]
E. puniceoluteum	52, **56**[6]
E. denticulatum	40, **52**[7]
E. flammeum	**50**[9]
E. fulgens	**24**[1,6]
E. cinnabarinum	**240**[2,4]
E. macrocarpum	**40**[10]
E. ibaguense	58, 70, 72, 76, 78[3,8,9]
E. flexuosum	**28**[3]

phylograms produced with BI in which accessions with longer branches were individually excluded in turn to determine if the exclusion results in changes in topology (Pelser *et al.*, 2010).

Assessment of ILS

To help distinguish between ILS (ancestral polymorphisms) or hybridization causing the plastid-nrDNA discordance, we implemented the approach of Pelser *et al.* (2010), which is based on the neutral premise that ancestral polymorphisms are likely to coalesce within five times the effective population size (N_e) generations (Rosenberg, 2003; Degnan & Rosenberg, 2009). Knowing the generation time for species, it is possible to estimate the minimum N_e to explain incongruence caused by ILS. It is estimated from each dated gene tree as divergence time of an incongruent clade/(5 × generation time), considering the 95% credibility interval. Finally, this interval of expected N_e is compared with an observed value for the species. If observed N_e < expected N_e, incongruence could be explained by ILS, but an older hybridization is not ruled out. On the other hand, if observed N_e > expected N_e, ILS is not an explanation for incongruence (Pelser *et al.*, 2010). Effectively, this test uses the estimated age of coalescence to distinguish between an older instance of incongruence probably caused by ancestral polymorphism from a more recent one that could only be caused by hybridization.

We co-estimated dated nrITS, plastid and species trees using the StarBEAST2 package v.0.15.11 (Ogilvie, Bouckaert & Drummond, 2017) implemented in BEAST v.2.6.3 (Bouckaert *et al.*, 2019). For this, we used the same species from the phylogenetic analysis, adding additional specimens (Supporting Information, Appendices S1 and S2) with *E. flexuosum* as the outgroup. Substitution rates were co-estimated using the *b*ModelTest package v.1.2.1 (Bouckaert & Drummond, 2017). We set analytical population size integration and birth–death process priors for the species tree estimation (Heled & Drummond, 2015). The clock was assumed to be relaxed with uncorrelated lognormal distributions for both datasets (Drummond *et al.*, 2006). Due to the lack of fossils, we calibrated our analysis using two secondary calibration points from Cardoso-Gustavson *et al.* (2018). We used normally distributed priors for the divergence between *E. flexuosum* and the ingroup (mean = 4.37 Mya, σ = 1.118) and for the ingroup node (mean = 3.67 Mya, σ = 0.9361; Cardoso-Gustavson *et al.*, 2018). We ran the analyses in two replicates of 25 million MCMC generations, sampling every 50 000 generations. Stationarity, effective sample size (ESS > 200) and convergence between runs were checked with Tracer v.1.7.1 (Rambaut *et al.*, 2018), and we discarded 5% of the first MCMC samples as burn-in. We combined the nrITS, plastid and species trees from the two runs using the LogCombiner post-processing tool (Bouckaert *et al.*, 2019), discarding the burn-in. Finally, maximum clade credibility (MCC) trees were obtained through the TreeAnnotator post-processing tool (Bouckaert *et al.*, 2019).

The observed N_e was independently estimated from nuclear DNA microsatellite data available from Pinheiro *et al.* (2016). This estimation was made for local populations of *E. secundum* from Nova Friburgo and Brejo da Madre de Deus and *E. xanthinum* from Nova Friburgo with the software NeEstimator v.2.1 (Do *et al.*, 2014), using the linkage disequilibrium method. Posteriorly, the observed N_e for each species was calculated as a product of the local N_e (for *E. xanthinum*) or mean of local N_e (for *E. secundum*) and the number of known locations for each species. *Epidendrum xanthinum* was recorded in only 17 locations, whereas *E. secundum* was documented in 773 sites from Mexico to Argentina (GBIF, 2021; Reflora, 2021; SpeciesLink, 2021). Although this multiplication is likely to result in overestimations of N_e, we are clearly working with conservative estimates of maximum population sizes. Finally, the observed plastid N_e was estimated as a quarter of the maximum microsatellite population size for each species because it is haploid and uniparentally inherited (Avise, 2009), and we assumed the most frequent nuclear ploidy of species is diploid (Assis *et al.*, 2013; Cordeiro 2019).

There are no studies that have estimated the generation time for species of *Epidendrum*. Based on the time of 2 years for the first flowering from seed observed in cultivated specimens of section

Amphyglottium (*E. radicans* × *E. xanthinum*) by Devadas, Medhi & Das (2010), we expect a generation time in natural conditions of around 4 years for *E. secundum* and *E. xanthinum*. To test for the robustness of our calculations, we used an expected N_e of 2, 4 and 8 years.

Cytogenetics

To reconstruct changes in basic chromosome numbers (from the literature, Table 1), ultrametric trees for both ITS and plastid datasets were produced using BEAST 1.8.0 (Drummond *et al.*, 2012). We applied a lognormal relaxed molecular clock model, following the Yule process prior, setting for 150 000 000 generations sampling every 1500 generations. The best-fitting nucleotide substitution models were the same as described above, and the remaining settings were the defaults. The results were evaluated using Tracer v.1.6, and MCC trees were constructed using TreeAnnotator v.1.10.4.

We reconstructed the ancestral states of basic chromosome numbers using ChromEvol v.2 (Mayrose, Barker & Otto, 2010; Glick & Mayrose, 2014) independently for nrITS and plastid trees. Chromosome numbers from areas described as hybrid zones were excluded from the analyses (Table 1). An ML approach was applied to test the number and directions of the changes in basic chromosome numbers across the phylogenetic trees. ChromEvol considers that chromosome number variation along the tree is a result of polyploidy (duplication events), demi-polyploidy (crossing between species with different ploidy), dysploidy (chromosome loss or gain events) or a combination of these events (Glick & Mayrose, 2014). We evaluated all models of chromosome number evolution available in ChromEvol. These estimates allowed us to infer the best evolutionary model to explain the variation in basic chromosome number under the AIC.

RESULTS

Phylogenetic reconstruction

For nrITS (Fig. 2A), the species of the Andean–Guyanan clade (*sensu* Pinheiro *et al.*, 2009; posterior probability 1.00, ML BP 100, parsimony BP 100; always in this order below) are strongly supported (1.00, 100, 100) as sister to the mostly well-supported Atlantic clade (0.94, 90, 56) including *E. secundum* and *E. xanthinum* in separate positions. The Atlantic clade is divided into two strongly supported subclades, one with *E. cinnabarinum* and *E. fulgens* (clade a, 0.99, 94, 75) and the other (clade b, 1.00, 99, 86) with *E. denticulatum*/*E. flammeum*/*E. xanthinum* (1.00, 98, 86) sister to *E. puniceoluteum*/*E. secundum* (1.00, 98, 98). *Epidendrum secundum* and *E. xanthinum* are here placed in strongly supported positions in two terminal clades nested among the species of the Atlantic clade. Multiple accessions of *E. xanthinum* and *E. secundum* from six populations in Brazil and one in Venezuela were included in a new nrITS analysis (Fig. 3), and the position of these species is the same as found previously (i.e. there is no variation in the nrITS allele recovered from multiple accessions of these two species; Fig. 2A).

In the plastid-based tree (Fig. 2B), the positions of *E. secundum* and *E. xanthinum* are completely different from those in the nrITS-based tree (Fig. 2A); they are well supported (1.00, 100, 100) as sister to the remaining species (1.00, 100, 100), which are divided into the same two strongly supported clades, the Andean–Guyanan (1.00, 100, 100) and Atlantic (1.00, 100, 95). In the latter, the positions of *E. fulgens* and *E. cinnabarinum* are unresolved, but *E. denticulatum*/*E. flammeum*/*E. puniceoluteum* (1.00, 97, 87) form a strongly supported but internally unresolved trichotomy. Except for the positions for *E. secundum* and *E. xanthinum*, results of the nrITS and plastid analyses (Fig. 2) produced nearly identical species placements with strong support. The only minor difference is that for nrITS *E. fulgens* and *E. cinnabarinum* are sister species in the Atlantic clade, whereas in the plastid-based tree their positions are unresolved in the Atlantic clade.

Reconstruction of basic chromosome numbers

Some species of the Atlantic clade exhibit constant chromosome numbers (Table 1), e.g. *E. fulgens* with $2n = 24$, *E. cinnabarinum* $2n = 240$ and *E. flammeum* $2n = 50$, whereas others are variable, putatively as a result of introgression in the well-known hybrid zones, e.g. *E. puniceoluteum* with $2n = 52$, 56 and *E. denticulatum* $2n = 38, 40, 52$. Conversely, *E. secundum* with $2n = 28, 30, 40, 42, 48, 50, 52, 54, 56, 58, 68, 80, 84$ and *E. xanthinum* with $2n = 28, 30, 40, 60, 80$ exhibit an extraordinary level of variation (Table 1).

We produced two independent chromosome number hypotheses for the plastid and nrITS trees (Fig. 4). The best chromosome evolution model according to AIC was LINEAR_RATE (five parameters: chromosome gain, linear chromosome gain, chromosome decrease, linear chromosome decrease and chromosome duplication rates) for both datasets (Supporting Information, Tables S1 and S2). The basic number $x = 14$ was suggested for the Atlantic (plastid, Fig. 4B) and Atlantic plus *E. secundum* and *E. xanthinum* clades (nrITS, Fig. 4A), but the probabilities for the nrITS results are all low compared to those for the plastid results.

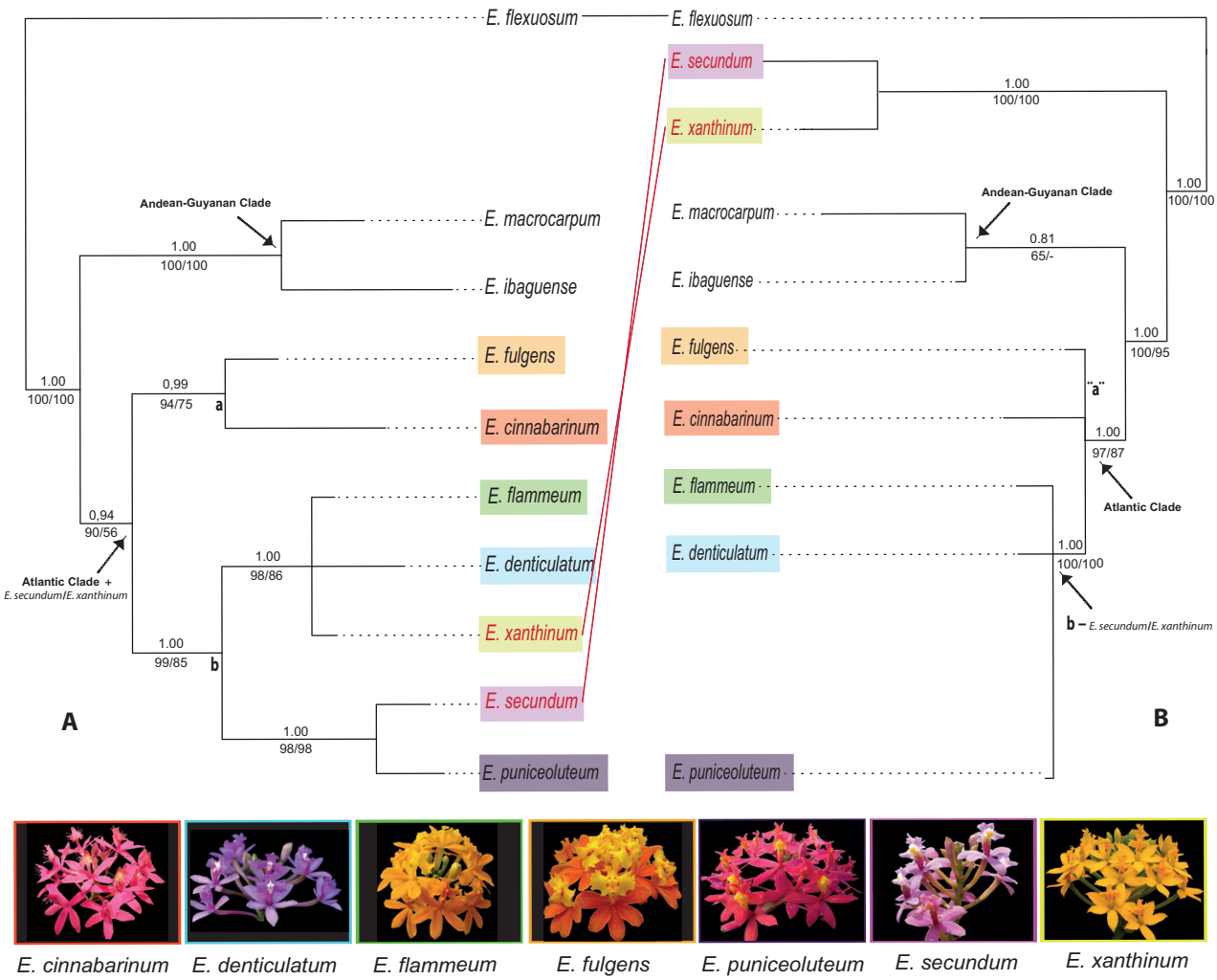

Figure 2. Phylogenetic relationships of the Brazilian species of *Epidendrum* subsections *Tuberculata* and *Carinata*. Bayesian inference of nuclear ribosomal ITS (A) and combined plastid (*matK*, *rpl32-trnL*, *trnL-trnF* and *trnT-trnL*) markers (B). Posterior probabilities are indicated above branches. Maximum-likelihood and maximum-parsimony bootstrap percentages are indicated below branches in this order separated by a slash.

For the plastid result, the model suggests that decreasing dysploidy (T^{ev} = 825.13) is more frequent than increasing dysploidy (T^{ev} = 404.95) and polyploidy (T^{ev} = 11.423). The main nodes of this tree retrieved an ancestral number of x_1 = 14, from which diverged *E. macrocarpum* and *E. ibaguense* through increasing dysploidy to x_2 = 20, *E. cinnabarinum* and *E. fulgens* through decreasing dysploidy to x_3 = 12, and *E. denticulatum* and *E. flammeum* through decreasing dysploidy to x_4 = 13. Polyploidy is frequent in terminals, such as *E. cinnabarinum* (n = 120 from x_3 = 12), *E. puniceolutteum* (n = 28 from x_1 = 14) and *E. denticulatum* (n = 26 from x_4 = 13), then followed by decreasing dysploidy as in *E. flammeum* (n = 25 from x_4 = 13).

Probabilities for the nrITS-based tree are < 20% (Supporting Information, Table S3), and we obtained similar results. Decreasing dysploidy (T^{ev} = 1041.98) was also more frequent than increasing dysploidy (T^{ev} = 414.61) in this analysis. This analysis retrieved x_5 = 14 for the clade formed by *E. secundum*, *E. puniceolutteum*, *E. denticulatum*, *E. flammeum* and *E. xanthinum*, which subsequently split into two subclades, *E. secundum* and *E. puniceolutteum* with x_6 = 16 through increasing dysploidy, and *E. denticulatum*, *E. flammeum* and *E. xanthinum* with x_7 = 13 though decreasing dysploidy. Dysploidy was also found to be frequent in the terminals, e.g. *E. macrocarpum* (n = 20, with increasing dysploidy from x = 19 + 1), *E. fulgens* (n = 12, decreasing dysploidy from x = 16 – 4), *E. secundum* (n = 14,

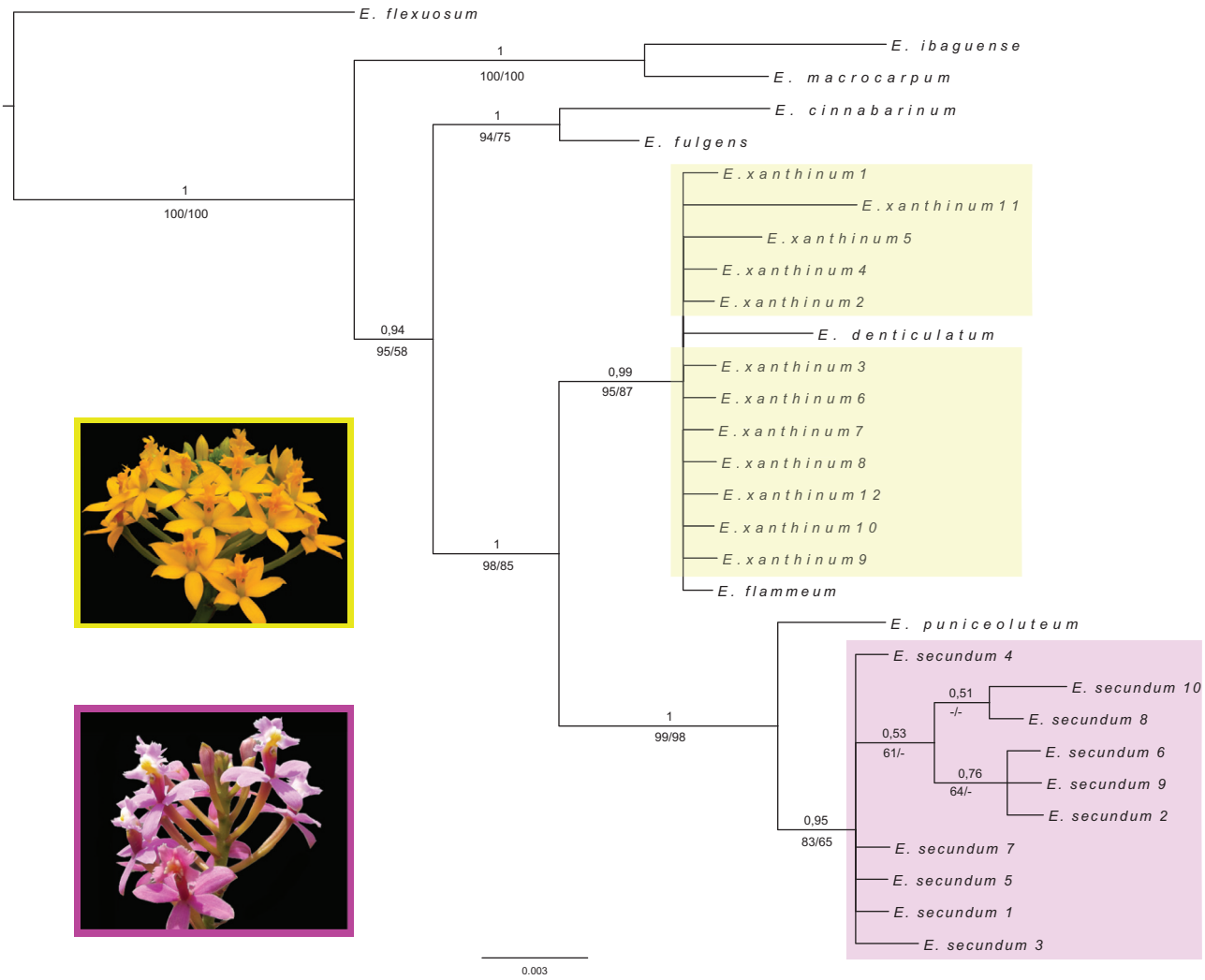

Figure 3. Phylogenetic relationships of the Brazilian species of *Epidendrum* subsections *Tuberculata* and *Carinata*, Bayesian inference of the nuclear ribosomal ITS from multiple specimens and provenances of *E. secundum* (pink) and *E. xanthunum* (yellow). Posterior probabilities are indicated above branches. Maximum-likelihood and maximum-parsimony bootstrap percentages are indicated below branches in this order separated by a slash. *Epidendrum secundum*: 1, Brazil, Santa Catarina; 2, 3, 5, Brazil, São Paulo; 4 Venezuela, Bolívar; 6, 7, Brazil, Minas Gerais, 8, 9, 10, Brazil, Rio de Janeiro. *Epidendrum xanthinum*: 1, 4, 5, 6, 10, Brazil, Minas Gerais; 2, 3, 7, 8, 9, 11, 12, Brazil, Rio de Janeiro.

decreasing dysploidy from $x = 16 - 2$) and *E. xanthinum* ($n = 14$, decreasing dysploidy from $x = 15 - 1$). The model also suggests an association of dysploidy and polyploidy in *E. ibaguense* (polyploidy and increasing dysploidy), *E. puniceoluteum* (polyploidy and decreasing dysploidy) and *E. flammeum* (polyploidy and decreasing dysploidy). A polyploid event is suggested for *E. denticulatum* ($n = 26$ from $x = 13$), and a demipolyploid event for *E. cinnabarinum* ($n = 120$ from $7.5\times$ $x = 16$, $f = 0.32$).

Assessment of ILS

Our estimation of divergence times suggests that plastid sequences of *E. secundum* and *E. xanthinum* coalesced *c*. 1.217 Mya [95% highest posterior density (HPD): 562 kya – 1.928 Mya] (Supporting Information, Fig. S1). The nrITS sequences of *E. secundum* and *E. puniceoluteum* coalesced *c*. 1.282 Mya (95% HPD: 525 kya – 2.074 Mya) (Fig. S2). For *E. xanthinum*, *E. denticulatum* and *E. flammeum*, nrITS sequences coalesced *c*. 1.206 Mya (95% HPD: 427 kya – 2.056 Mya) (Fig. S2).

Observed N_e was 72.5 and 70.0 for *E. secundum* from Nova Friburgo and Brejo da Madre de Deus, respectively. For *E. xanthinum* from Nova Friburgo, observed N_e was 59.5. Therefore, the maximum observed N_e for nrITS was 55 076.25 and 1011.50 for *E. secundum* and *E. xanthinum*, respectively. For plastid

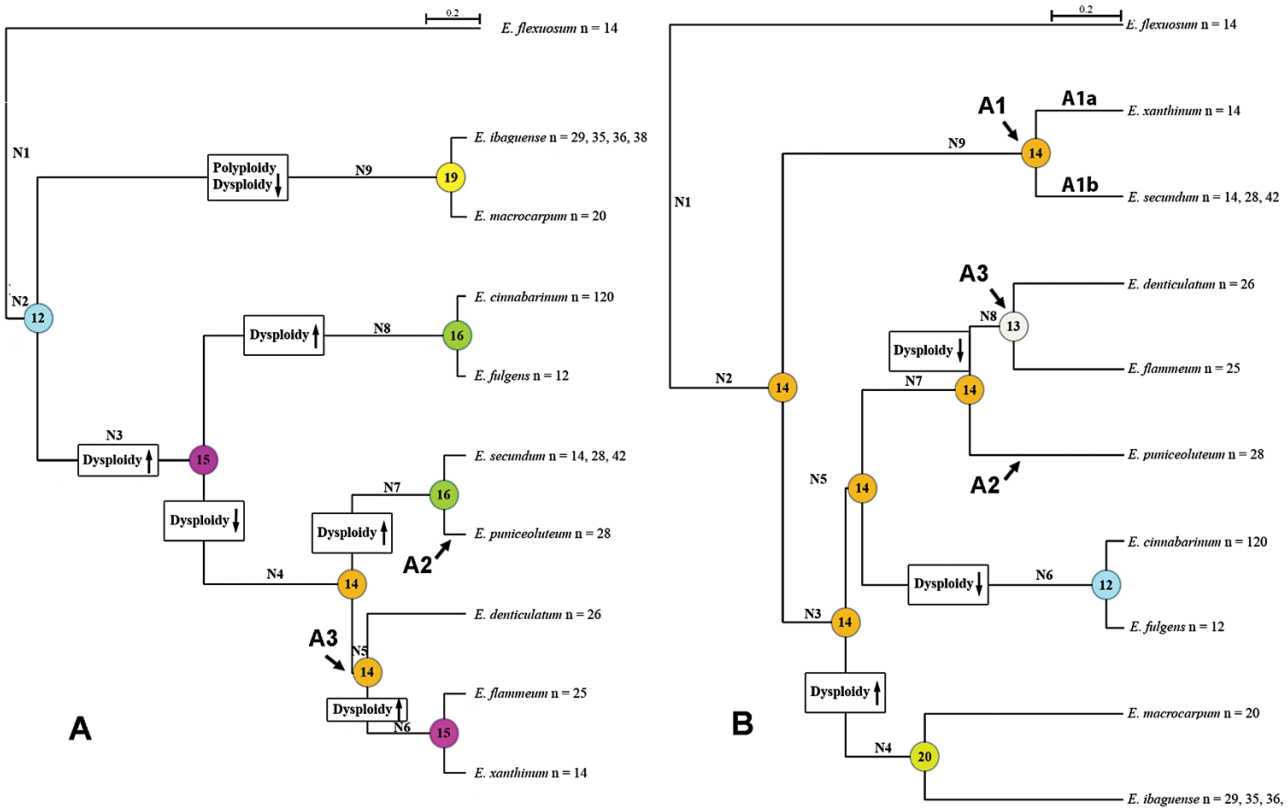

Figure 4. Reconstruction of chromosome number evolution in the Atlantic clade of *Epidendrum* with the linear_rate model implemented in ChromEvol 2.0. A, tree for nr TS; B, plastid data tree. N followed by cardinal numbers represents each node of the tree. The numbers inside the coloured circles represent the most likely ancestral chromosome number for each node. The main chromosome number changes involved in a group of species is represented inside rectangles (e.g. increasing, decreasing dysploidy). The most likely ancestral chromosome numbers are given in Supporting Information Table S3.

genomes, the maximum observed N_e was 13 769.06 and 252.88 for *E. secundum* and *E. xanthinum*, respectively. Estimated N_e was always greater than observed N_e for *E. xanthinum* and greater than observed N_e for *E. secundum* plastid data, rejecting ILS as an explanation for the incongruence (Table 2). On the other hand, observed N_e for the *E. secundum* nrITS data was within the ranges of estimated N_e, except for nrITS in the scenario with an 8-year generation time, in which observed N_e was greater than the expected interval of N_e (13.10–51.90 vs. 55.07). Specifically in this case, ILS is the most plausible hypothesis to explain the incongruence (Table 2), whereas neither ILS nor ancient gene flow can be rejected as explanations for incongruence in *E. secundum* nrITS for generation-time scenarios of 2 and 4 years.

DISCUSSION

Discordance between gene trees has historically challenged phylogenetic analysis and, consequently, classifications derived from such efforts (Knapp, Chase & Clarkson, 2004; Cox *et al.*, 2014; Ruprecht *et al.*, 2017). However, recent developments in the field do not interpret incongruent results as noise, but instead as windows of opportunity to study the heterogeneous impact of evolution on particular genomic regions and organelles (Dodsworth *et al.*, 2020; Stubbs *et al.*, 2020; Dong *et al.*, 2021). In this context, hybridization has been pointed out as an important source of phylogenetic incongruence, with ILS, horizontal gene transfer, and gene duplication and loss (Knowles *et al.*, 2018). Some of these processes, such as hybridization and gene duplication/loss, may result in chromosome number changes. Thus, the study of species with variable chromosome numbers has great potential for revealing the sources of discrepancy in phylogenetic trees (Yamagishi-Costa, 2009; Mckain *et al.*, 2012; Weiss-Schneeweiss & Schneeweiss, 2013; Nollet *et al.*, 2017; Garcia *et al.*, 2018). Our results identified a basic number $x = 14$ for this group of *Epidendrum*, suggesting that major karyotype changes occurred

Table 2. Results of estimated N_e calculated for *Epidendrum secundum* and *E. xanthinum*, two species with plastid–nuclear discordance (see Results). Complete coalescence was assumed to occur within N_e generations, and calculations were performed for generation times of 2, 4 and 8 years

Incongruent lineage	Data set	Duration of putative ILS (Mya)	Estimated N_e (×1000) for assumed generation times			Observed N_e (×1000)
			2 years	4 years	8 years	
E. secundum	Plastid	0.562–1.928	56.2–192.8**	28.1–96.4**	14.1–48.2**	13.8
	ITS	0.525–2.074	52.5–207.4	26.3–103.7	13.1–51.9*	55.1
E. xanthinum	Plastid	0.562–1.928	56.2–192.8**	28.1–96.4**	14.1–48.2**	0.25
	ITS	0.427–2.056	42.7–205.6**	21.4–102.8**	10.7–51.4**	1.0

*Estimated N_e < observed N_e, **estimated N_e > observed N_e.

after species divergence in nearly all cases, and many ancestral karyotypes were unchanged. Chromosome gains, polyploidy and decreasing dysploidy were the most frequent changes estimated for these species. In addition, based on our ILS testing, hybridization is most probably the driver that played an important role in increasing chromosome number variation in *E. xanthinum* (all cases) and *E. secundum* (plastid data). Moreover, ILS is not expected to produce such low levels of divergence, as observed in the nrITS of *E. xanthinum* and *E. flammeum*/*E.denticulatum* in one case and *E. secundum* and *E. puniceoluteum* in the other (Fig. 2). ILS or ancestral polymorphisms are due to phenomena that occurred in the common ancestor of the modern species, so these sequences inherited independently from this ancestor should be highly divergent (Dodsworth *et al.*, 2020). This difference in divergence between and old and much more recent events is the basis of the Pelser *et al.* (2010) test, which cannot, however, distinguish between ILS and ancient hybridization. By combining multiple sources of information, our study has provided insights into phylogenetic discordance in *Epidendrum* and revealed the most plausible mechanisms responsible for shaping chromosome number variation.

Evidence from previous studies of *Epidendrum* suggested that hybridization and introgression occur in species of the Atlantic clade (Pinheiro *et al.*, 2009). Introgression was identified in separate sympatric populations of *E. fulgens* ($2n = 24$) and *E. puniceoluteum* ($2n = 56$; both *E.* subsection *Carinata*), in which hybrids with intermediate chromosome numbers were found (Pinheiro *et al.*, 2010; Moraes *et al.*, 2013). The identification of additional hybrid zones composed of different parental species, such as *E. fulgens* and *E. denticulatum* ($2n = 52$; also *E.* subsection *Carinata*), and *E. orchidiflorum* ($2n = 156$) confirms that major differences in chromosome number do not necessarily preclude hybridization in this group (Pinheiro *et al.*, 2015; Arida *et al.*, 2021). However, in these studies, variation in parental chromosome numbers was not detected (Table 1), suggesting that introgression had only a limited potential to alter chromosome numbers.

A different scenario was proposed for *E. secundum* (*E.* subsection *Tuberculata*), because polyploidy was observed throughout its distribution. For example, there are allopatric populations with $2n = 28$ (Bolivia and Venezuela), 56 (south-eastern Brazil) and 84 (north-eastern Brazil), and high rates of dysploid chromosome variation have been observed in *E. secundum* when in sympatry with other species (Assis *et al.*, 2013; Nollet *et al.*, 2022). Karyological evidence suggested that *E. secundum* and *E. xanthinum* have $x = 14$ as the basic chromosome number with $2n = 28$ (Assis *et al.*, 2013; Nollet *et al.*, 2022). Furthermore, substantial variation in CMA/DAPI dye banding is observed in these two species, and B chromosomes have also been recorded (Nollet *et al.*, 2022), providing clear support for characterization of their karyotypes as unstable.

Species with unstable karyotypes are probably the result of neo- or palaeo-allopolyploid events (Murray & Young, 2001; Chester *et al.*, 2012; Souza *et al.*, 2012; Pelé, Rousseau-Gueutin & Chèvre, 2018; Prančl *et al.*, 2018; Mason & Wendel, 2020). However, there is no evidence of recently formed hybrids or current hybrid zones between *E. secundum* and *E. puniceoluteum*, which are sister species according to the nrITS tree, or between *E. xanthinum*, *E. denticulatum* and *E. flammeum* (Fig. 2A). These putative hybridization events probably occurred at least 3.7 Mya according to Cardoso-Gustavson *et al.* (2018).

It is possible that these two species originated as a result of hybridization involving the common ancestor of *E. secundum* and *E. xanthinum*, a now extinct species, here labelled as ancestor 1 (A1, $x = 14$, Fig. 4B), which bred with the ancestor of *E. puniceoluteum*, here ancestor 2 (A2, $x = 14$, Fig. 4A) and that of *E. denticulatum* and *E. flammeum*, ancestor 3 (A3, $x = 13$, Fig. 4A, B). A1 could also be interpreted as the common ancestor of all species in *E.* subsection *Tuberculata* (*sensu* Brieger 1976–1977).

Given that plastid DNA and nrITS represent just two alleles in the genomes of these species and are not necessarily indicative of 50:50 contributions from each ancestral parent, it is equally plausible that for both *E. secundum* and *E. xanthinum*, there were two introgressive events not at their origin but rather in the individual ancestors of these species after divergence from their common ancestor, when they could be considered *Epidendrum* 'proto'-*secundum* and *Epidendrum* 'proto'-*xanthinum*. These events resulted in capture of the nrITS sequences found in the ancestors of *E. flammeum*/*denticulatum* and *E. puniceoluteum* (Atlantic clade), respectively. At the same time as capture of the rDNA took place, introgression of repetitive elements (including retrotranspons) subsequently infiltrated the rest of the native nuclear genome, creating novel chromosomal hotspots (Van der Knaap *et al.*, 2004) that facilitated multiple rearrangements, leading to the modern dysploid variation observed in chromosome numbers in *E. secundum* and *E. xanthinum*.

Based on the results, we hypothesize that palaeo-hybridization events were unidirectional towards A1 or A1a and A1b, similar to what has been observed in current hybrid zones in the group (Pinheiro *et al.*, 2010; Moraes *et al.*, 2013). In *E. secundum* and *E. xanthinum*, A1/ or A1a/A1b were the maternal parents, whereas A2 and A3 were the paternal ones, respectively. The basic numbers reconstructed for the ancestral species support the possibility of hybridization (Fig. 4). Thus, past hybridization/introgression perhaps represents a key component for explaining the incongruence between plastid and nuclear datasets for some groups of species in *Epidendrum* (Klein *et al.*, 2019; Mendoza *et al.*, 2020; Pessoa *et al.*, 2021). Unstable karyotypes are not common in the genus, but in other species-rich and similarly complex groups, such as *E. nocturnum*, a similar pattern of great variation in chromosome numbers is observed (Felix & Guerra, 2010; Assis *et al.*, 2013; Cordeiro, 2019). Thus, past hybridization is probably not the only explanation for the large number of species in *Epidendrum*, but it is probably one of the contributing factors.

The phylogenetic positions of *E. denticulatum*, *E. flammeum* and *E. puniceoluteum* are congruent in the nrITS and plastid trees (Fig. 2). Sympatric populations of *E. denticulatum* and *E. secundum* were studied by Vieira *et al.* (2017) to investigate hybridization, and successful artificial crosses demonstrated that these species are cross-compatible and share pollinators. However, no hybrids have been identified based on morphology or genetic data, and Vieira *et al.* (2017) suggested that there is no current gene flow.

Extinct species occasionally hybridized in the past with direct ancestors of others, resulting in introgression and creation of the species we observe today, a phenomenon that can be detected by discordant phylogenetic signature (Ottenburghs, 2020). Hybridization increases the number of taxa via intermixing, which could partially help to explain the enigma of some highly diverse genera, such as *Epidendrum*. Although examples of phylogenetic incongruence between nuclear and plastid datasets are known for several *Epidendrum* spp. (Klein *et al.*, 2019; Mendoza *et al.*, 2020; Pessoa *et al.*, 2021), it is the first time that the consequences of past hybridization/introgression have been documented using a combination of cytogenetic and molecular phylogenetic/population data (Pinheiro *et al.*, 2009, 2015; Felix & Guerra, 2010; Pessoa *et al.*, 2012; Assis *et al.*, 2013; Moraes *et al.*, 2013; Vieira *et al.*, 2017; Cardoso-Gustavson *et al.*, 2018; Nollet *et al.*, 2022). Our results suggest that past hybridization may have shaped the extreme chromosome number differences detected in *Epidendrum*. Even when hybrids are mostly sterile, vegetative propagation allows persistence of hybrid genotypes at sympatric sites, increasing the opportunity for rare seed set, interspecific genetic exchange and recombination (Pinheiro & Cozzolino, 2013; Arida *et al.*, 2021).

Considering that *Epidendrum* is one of the largest orchid genera in the tropics, occurring in many habitat types (sand dune vegetation, rock outcrops, swamps, cloud forests, etc.), additional studies of chromosome numbers among and within species will clarify the potential role of chromosome changes in local adaptation. Additional chromosome counts are also needed to help explain phylogenetic discordance. Indeed, there is an urgent need to build a representative phylogenetic tree for the genus to aid in determining whether gene-tree discordance is restricted to specific genomic regions (Gori *et al.*, 2016; Knowles *et al.*, 2018). By embracing the heterogeneity in gene trees and exploring sources of discord, we stand to gain a better understanding of the mechanisms associated with divergence leading to speciation. Thus, we encourage similar studies using genomic and chromosome data that will provide a better understanding of the evolutionary dynamics of this genus. Further studies including a larger number of species using techniques that sample much more of the nuclear genome could evaluate whether gene flow and karyotype variability are associated with increased speciation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Divergence time estimates of the Brazilian species of *Epidendrum* section *Amphiglottium* subsections *Tuberculata* and *Carinata* based on the ITS dataset. Bars represent 95% highest posterior density (HPD) estimates. A timescale is given at the bottom.

Figure S2. Divergence time estimates of the Brazilian species of *Epidendrum* section *Amphiglottium* subsections *Tuberculata* and *Carinata* based on the plastid dataset (*matK*, *rpl32-trnL*, *trnL-trnF* and *trnT-trnL*). Bars represent 95% highest posterior density (HPD) estimates. A timescale is given at the bottom.

Table S1. Log-likelihood and Akaike information criterion (AIC) score estimates for the plastid dataset analysed by ChromEvol software.

Table S2. Log-likelihood and Akaike information criterion (AIC) score estimates for the ITS dataset analysed by ChromEvol software.

Table S3. Most likely ancestral chromosome number for each node according to ChromEvol.

Appendix S1. GenBank accession numbers used in the analyses. Voucher information can be found in Pinheiro *et al.* (2009)[1], Pessoa *et al.* (2012)[2], Cardoso-Gustavson *et al.* (2018)[3], Vieira *et al.* (2017)[4], Lahayne *et al.* (2008)[5] and Appendix S2[6]. Bold: specimens used in the datasets including one terminal of each species.

Appendix S2. Voucher information for the sequences newly produced for this study.