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Using multiple analytical methods to improve phylogenetic hypotheses in *Minaria* (Apocynaceae)

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ABSTRACT

Metastelmatinae is a neotropical subtribe of Asclepiadoideae (Apocynaceae), comprising 13 genera and around 260 species whose phylogenetic relationships are often unresolved or incongruent between plastid and nuclear datasets. The genus *Minaria* is one of the first lineages to emerge in the Metastelmatinae and is highly supported based on plastid markers. It comprises 21 species, most of which are endemic to small areas with open vegetation in the Espinhaço Range, Brazil. In the work presented here, we use plastid (*rps16, trnH–psbA, trnS–trnG*, and *trnD–trnT*) and nuclear (ITS and ETS) datasets to investigate the relationships within *Minaria*. We show that the three methods mostly used in phylogenetic studies, namely, maximum parsimony, maximum likelihood, and Bayesian Inference, have different performances and that a pluralistic analytical approach combining results from them can increase tree resolution and clade confidence, providing valuable phylogenetic information.

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1. Introduction

Metastelmatinae is a neotropical subtribe, comprising 13 genera and nearly 260 species. The monophyly of most genera of Metastelmatinae has been questioned by phylogenetic studies, but a generic re-circumscription has not been possible because the relationships within the subtribe are often unresolved, phylogenetic signals are incongruent between plastid and nuclear datasets, and clades usually lack diagnostic morphological features (Silva et al., 2012). In spite of this difficulty, Minaria has been identified as one of the first lineages to diverge in the Metastelmatinae (e.g., Rapini et al., 2006). The genus was not recognized based on morphology, but is highly supported in phylogenetic studies based on the plastid trnL-F intergenic spacer and trnL intron (Rapini et al., 2003, 2007) and was confirmed by subsequent analyses with the additional plastid markers trnT-L and trnS-G intergenic spacers, rps16 intron, and part of matK gene (Liede-Schumann et al., 2005; Rapini et al., 2006).

Originally, *Minaria* comprised 19 species that are mostly endemic to small areas with open vegetation in the Espinhaço Range, in the states of Minas Gerais, in eastern Brazil (Konno et al., 2006). Nevertheless, a study comprising 25% of the Metastelmatinae (Silva et al., 2012) suggested that two other species also endemic to the Espinhaço Range, but in the state of Bahia, should also be included in *Minaria* and contradicted the position of *Minaria*

* Corresponding author. *E-mail address:* rapinibot@yahoo.com.br (A. Rapini). *polygaloides* in the genus. These relationships were not resolved with ITS, but were strongly supported based on plastid dataset and plastid and ITS combined analyses.

Due to the distribution of *Minaria* spp., most of which are rare and/or narrowly distributed in the Espinhaço range, the phylogenetics of the genus may reveal biogeographic patterns and help to assess priority areas for conservation in the region (Ribeiro et al., 2012), besides questions about the origin and maintenance of endemism. Therefore, the present work has three primary objectives: (1) to evaluate the circumscription of *Minaria*, (2) to position the genus in the Metastelmatinae, and (3) to establish the relationships among its species. We present the results produced by the three most popular analytical methods in phylogenetic studies – maximum parsimony, maximum likelihood, and Bayesian Inference – and compare their performances. Finally, we show that combining results obtained by different methods can increase clade confidence and the resolution of our phylogenetic hypotheses.

2. Materials and methods

We sampled 41 species of Metastelmatinae, concentrating the sampling in *Minaria*, with 18 species (Appendix). Three specimens of *M. cordata* were sampled to include the whole variation of the species, which is divided into varieties (Fontella-Pereira, 1989). The tree was rooted in *Blepharodon ampliflorum* because it is closely related to *B. lineare* (and eventually considered a synonym of this species; e.g., Morillo, 1976; Fontella-Pereira et al., 1984),

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which was shown to be sister to the remaining Metastelmatinae (Liede-Schumann et al., 2005; Rapini et al., 2006).

DNA was extracted from dehydrated leaves using the 2 × CTAB protocol (Doyle and Doyle, 1987), adapted for microtubes. We tested 25 regions suggested as potentially useful in phylogenetic reconstructions at the species level. Four plastid (*rps16* intron, *trnH–psbA*, *trnS–trnG* and *trnD–trnT* intergenic spacers) and two nuclear regions (ITS and ETS) were selected based on their variability and number of parsimony-informative characters (Table 1). The amplification mix that achieved success for most regions consisted of 1 µL total DNA, 1 × buffer, 2.0 mM MgCl₂, 0.2 mM dNTP, 0.2 mM primer, 10 ng BSA, *Taq* DNA polimerase (Phoneutria) – 1.25 units for the plastid regions and 0.75 for the nuclear ones – complemented with ultrapure water to 25 µL; for ITS and ETS amplification, 1.0 M betaine and 2% DMSO were added to the mix. TopTaq (Quiagen) mix was used to amplify regions that failed with the prepared mix, following the standard protocol in the kit manual.

PCR products were purified in EXO-SAP (Amersham Biosciences) enzymatic reactions or using PEG (polyethylene glycol), and were sequenced directly with the same primers used for the PCR amplification. Sequence eletropherograms were produced in an automatic sequencer (ABI 3130XL Genetic Analyzer) using Big Dye Terminator 3.1 (Applied Biosystem). They were edited using the Staden Package (Staden et al., 2003) and aligned using BioEdit Sequence Alignment Editor (Hall, 1999). Whenever we found polymorphic paralogues of ITS, which was the case in Minaria grazielae, M. hemipogonoides, M. polygaloides, M. semirii, and Blepharodon pictum, we cloned the PCR products. For cloning, we used the pGEM[®] -T kit, following the manufacturer's (Promega) protocols. At least five colonies were sequenced for cloned PCR product. Clones of the same species formed supported clades (not shown), suggesting they coalesce within the respective species. Therefore, only one sequence per species was included in the final analyses.

Plastid and nuclear datasets were analyzed in combined (total evidence) and separate analyses. We analyzed the datasets with the three most common phylogenetic methods: maximum parsimony (MP), and two model-based methods, Bayesian Inference (BI) and maximum likelihood (ML). To evaluate their performances with empirical data is especially difficult because the true phylogeny is unknown. In spite of that, while incongruence between plastid and nuclear datasets might be produced by biological and stochastic processes (Madisson, 1997; Degnan and Rosenberg, 2009), the simplest explanation for their congruence is a common history representing species relationships. Therefore, clades supported independently by the plastid and nuclear datasets are treated as "correct" for the purpose of the present study. Clades with 95% accuracy were considered supported. Therefore, we constrained our supported clades to those either with Bayesian posterior probability (PP) $\ge 95\%$ or with non-parametric bootstrap support $(BS) \ge 75\%$ (being conservative). The former measure is considered a better estimator of accuracy, and $BS \ge 70\%$ usually corresponds to $PP \ge 95\%$ (Hillis and Bull, 1993; Alfaro et al., 2003). Without the root, a fully resolved tree with 41 terminals, as in our analyses, presents 38 clades. The efficiency of each method was then measured by the percentage of correct clades recovered with accuracy based on the same dataset.

The MP analyses were conducted in PAUP v. 4.0b10a (Swofford, 2000), with characters treated under equal weights and unordered. Heuristic searches were performed with tree-bisection and reconnection (TBR) branch swapping on 1000 random-taxon addition replicates, holding no more than 20 trees per replicate; the trees saved were then subject of another set of TBR swapping with a limit of 20,000 trees. The confidence of the clades was statistically evaluated by non-parametric bootstrap support (MP_BT) (Felsenstein, 1985) calculated through 1000 pseudo-replicates of simple-taxon addition, followed by TBR swapping, keeping a limit of 15 trees per pseudo-replicate.

Table 1

Regions of the plastidial and nuclear genome tested for *Minaria* species, its success in amplification (Ampl.) and sequencing (Seq.). Quantitative traits evaluated for choice of markers: L = length of the aligned sequences; Indels = gaps; Subst. = number of nucleotide substitutions; Inv = inversions; PIC = potentially informative characters; % variation = variability: sequence length ratio; IP = parsimony informative characters; and N = number of species. *Amplification in insufficient quantity of DNA for sequencing reaction. *Sequences not used. Regions selected for phylogenetics analysis are in bold.

Regions	Reference	Ampl.	Seq.	<i>L</i> (pb)	Indels	Subst	Inv	PIC	% Variation	IP	Ν
Plastidial											
psbA-trnH	Hamilton, 1999; Shaw et al., 2005	Yes	Yes	436	3	10		13	2.98	4	8
rpoB-trnC	Shaw et al., 2005	Yes	Yes	1326	3	12		15	1.13	3	7
trnD-T	Demesure et al., 1995; Shaw et al., 2005	Yes	Yes	1144	10	1		11	0.96		3
atpF-H	Barcode Kew	Yes	Yes	555	1	3		4	0.72	0	4
psbK-I	Barcode Kew	Yes	Yes	240	1			1	0.42		5
rps16	Oxelman et al., 1997; Shaw et al., 2005	Yes	Yes	845	6	12		18	2.13	2	7
trnL-F	Taberlet et al., 1991; Shaw et al., 2005	Yes	Yes	936	10	9		19	2.03	0	9
trnS-G	Shaw et al., 2005	Yes	Yes	671	5	12	73	90	13.41	3	8
trnQ–rps16	Shaw et al., 2007	Yes	Yes#								4
rps16–trnK	Shaw et al., 2007	Yes	Yes [#]								4
rpl32–trnL	Shaw et al., 2007	Yes	Yes [#]								5
trnV–ndhC	Shaw et al., 2005	No	No	-							
ndhF–rpl32	Shaw Shaw et al., 2005	No	No	-							
psbD–trnT	Shaw et al., 2007	Yes	Yes	1297	2	1		3	0.23		3
psbJ–petA	Shaw et al., 2007	Yes	Yes	738	2	2	18	22	2.98	0	7
atpI–atpH	Shaw et al., 2007	Yes	Yes	923	2	3		5	0.54		3
Nuclear											
ITS	White et al., 1990; Sun et al., 1994	Yes	Yes	782	60	32		92	11.76	16	7
ETS	Beardsley and Olmstead, 2002; Baldwin and Markos, 1998	Yes	Yes	516	42	19		61	11.82	4	8
GBSSI2-8	Peralta and Spooner, 2001; Levin and Miller, 2005	Yes	Yes	1097	9	26		34	3.10	3	4
GBSSI8-12	Winkworth and Donoghue, 2004	No	No								
agt1	Li et al., 2008	Yes	Yes#								
at103	Li et al., 2008	No	No								
eif3E	Li et al., 2008	No	No								
aroB	Li et al., 2008	Yes*	No								
sqD	Li et al., 2008	No	No								

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