Danilo Soares Gissi

Phylogeny of *Phlegmariurus*
(Lycopodiaceae) focusing on Brazilian
endemic species

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Filogenia de *Phlegmariurus* (Lycopodiaceae) com ênfase em espécies endêmicas do Brasil

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Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Mestre em Botânica, na Área de Sistemática, Evolução e Biogeografia de Plantas Vasculares.

Orientador: Dr. Jefferson Prado

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Prof. Dr. Jefferson Prado

Orientador(a)

Dedico a todas as pessoas,
nas quais eu sou capaz de por
um sorriso.

We all think we are going to be great and we feel a little bit robbed when our expectations aren't met but sometimes our expectations sell us short. Sometimes the expected simply pails to the unexpected. You gotta wonder why we cling to our expectations. Because the expected is just what keeps us steady, standing, still. The expected is just the beginning. The unexpected is what changes our lives.

- Meredith Grey
(Season3, Ep.13-Great Expectations)
Grey's Anatomy.

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Contents

Introduction

According to phylogeny works (Pryer *et al.*, 2004; Smith *et al.*, 2006; Smith *et al.*, 2008), there is a common agreement that a phylogenetic dichotomy separates the vascular plants into modern lycophytes and euphyllophytes (ferns and seed plants). Lycophytes comprise three families: Lycopodiaceae, with plants homosporous and eligulated, Isoetaceae and Selaginellaceae, both heterosporous and ligulated plants.

The family Lycopodiaceae does not stand out economically as other vascular plants. However, due to the presence of highly flammable compounds in the walls of spores, they have been used by magicians and sorcerers of the Middle Ages and like flash powder in old cameras. Several lycopod alkaloids, such as Huperzine have been used as drugs against Alzheimer's disease (Tang & Han, 1999; Olafsdóttir *et al.*, 2013; Murphy & Sarpong, 2014). *Phlegmariurus saururus* (Lam.) B. Øllg. has an extensive ethnopharmacological use, mainly because of its aphrodisiac properties that has been proven by Hnatyszyn *et al.,* (2003) and Birri *et al.,* (2014). Diacetyl sauroine (DAS) was obtained and its chemical structures were analyzed and have been demonstrated to improve learning abilities of mice. DAS, therefore, may be a promising compound as a nootropic therapeutic drug (Vallejo *et al.*, 2014). The Lycopodiaceae species, especially *Phlegmariurus* (Herter) Holub have high ornamental potential that is little explored.

The family Lycopodiaceae

Lycopodiaceae present cosmopolitan distribution and they have nearly

500 species (Øllgaard, 1987). W. G. Herter (1909), G. G. Herter (1949, 1950) and Nessel (1927, 1955) proposed the most detailed systems for the family Lycopodiaceae under only the genus *Lycopodium* or both *Lycopodium* and *Urostachys*, respectively. Both Herter and Nessel acknowledged too many species, even committing errors or accepting the superfluous name *Urostachys* for the *Huperzia* species. Most of the groups proposed for the Huperzioid species were considered unnatural and challenging to be morphologically recognized.

Tryon & Tryon (1982) have recognized only one large genus (*Lycopodium* L.) in order to group all species, except *Phylloglossum* Kunze. Meanwhile, Øllgaard (1987, 1992, 1994) and Øllgaard & Windisch (1987) considered three genera for the Neotropics: *Huperzia* Bernh., *Lycopodium* and *Lycopodiella* Holub. These genera were divided into informal groups, which represent the genera proposed by Holub (1964). W. G. Herter (1923) has elevated *Lycopodium* subg. *Urostachys* Herter to a generic level representing the genus *Huperzia*. The name *Huperzia* Bernh. (1801) is older than *Urostachys* (Herter) Herter, thus the last is an illegitimate superfluous name.

Holub (1964) has segregated *Phlegmariurus* from *Huperzia* and divided *Lycopodium* in *Austrolycopodium* Holub, *Diphasiastrum* Holub, *Diphasium* Rothm., *Lycopodium s.str.*, *Lycopodiastrum* Holub, *Pseudodiphasium* Holub e *Pseudolycopodium* Holub. He also treated *Lycopodiella* on four genera: *Lycopodiella s.str.*, *Palhinhaea* Vasc. & Franco, *Pseudolycopodiella* Holub, and *Lateristachys* Holub. Haines (2003) described *Spinulum* Haines and *Dendrolycopodium* Haines segregated from *Lycopodium* sensu Øllgaard (1987) and considered *Huperzia*, *Phlegmariurus*, and *Phylloglossum* as a distinct family: Huperziaceae Rothm.

Although the genus *Phlegmariurus* has been described by Holub (1964)

2

to accommodate some species of *Huperzia*, the genus was not accepted by several authors (e.g., Øllgaard 1987, 1992; Øllgaard & Windisch, 1987; Ramos & Sylvestre, 2010). More recently, including Christenhusz *et al.,* (2011) who, presenting no new molecular data, included *Phylloglossum* within *Huperzia* in order to recognize the monophyly of this genus.

Recent molecular studies (Field & Bostock, 2013; Field *et al.*, 2016) have proposed sixteen genera for the family, with easy separation comprised into two subfamilies Lycopodioideae and Huperzioideae *sensu* Wagner & Beitel (1992). Lycopodioideae is characterized by anisostomous heteroblastic branching, determinate strobili, a broad stem stele with the absence of corticular root emergence, leaves with basal mucilage canals, new shoots arising adventitiously from lateral branches, sporophylls peltate or nearly peltate, deciduous, reticulate—rugate spores, erect gametophytes and the absence of paraphyses among gametangia. Huperzioideae, on the other hand, is characterized by isostomous branching, corticular emergence of roots, new shoots arising axiliary from tufted shoot bases, tufted root system, paleate sporophylls, sporophylls evergreen lacking a mucilage duct in the sporophyll base, strictly reniform axiliary sporangia, absence of a sporangial stalk, foveolate-fossulate spores, dorsiventral gametophytes with a horizontal growth axis, and the presence of paraphyses among the gametangia (Wagner & Beitel, 1992; Field *et al*., 2016).

Lycopodioideae can be divided into two clades corresponding to the genera *Lycopodium* and *Lycopodiella* sensu Øllgaard (1987) or the subfamilies Lycopodioideae and Lycopodielloideae sensu Øllgaard (2015). Lycopodioideae sensu Øllgaard (2015) differs from the other subfamily by shoots inclined, spore sculpture reticulate, scabrate or baculate; capsule wall cells sinuate to invaginate; root stele like

rhizome stele at base; gametophyte subterranean, non-photosynthetic, growing by a ring meristem; archegonia long, persistent. It is represented by the genera *Austrolycopodium*, *Dendrolycopodium*, *Diphasium*, *Diphasiastrum*, *Lycopodium s.str.*, *Lycopodiastrum*, *Pseudodiphasium*, *Pseudolycopodium*, and *Spinulum*. Lycopodielloideae present shoots flabellate, spore sculpture rugulose; capsule walls straight; root stele various; gametophytes subsurficial, with photosynthetic lobes; archegonia short, ephemeral. It is represented by the genera *Lateristachys, Lycopodiella, Palhinhaea,* and *Pseudolycopodiella.*

In the subfamily Huperzioideae three distinct genera are recognized: *Phylloglossum*, *Huperzia,* and *Phlegmariurus* (Øllgaard 2012a, b; Field & Bostock, 2013; Zhang & Iwatsuki, 2013, Field *et al.*, 2016)

Phylloglossum is a monotypic genus (*P. drummondii* Kunze), with occurrence restricted to Australia and New Zealand (Øllgaard & Windisch, 1987) and it is distinguished by the presence of basal microphylls produced from an underground tuber and the aphyllous strobilus peduncle (Haines, 2003).

Huperzia and *Phlegmariurus* share several characters such as isotomously branched stems, roots forming a basal tuft, sporophylls persistent and photosynthetic after dehiscence of the sporangia located in the apical part of the stem and not gathered in strobili. However, the genus *Huperzia* consists exclusively of terrestrial plants, with bulbils produced by buds and spores with concave sides at the equator and truncated angles. Nonetheless, *Phlegmariurus* are epiphytic or terrestrial plants that show no bulbils and have spores with convex sides at the equator and obtuse or acute angles (Haines, 2003; Arana & Øllgaard, 2012). *Huperzia* occurs mainly in temperate regions and has 55 species, of which 27 spp. (18 endemics) occur in China

4

(Zhang & Iwatsuki, 2013), whereas *Phlegmariurus* features 114 spp. in the Paleotropics (Field & Bostock, 2013) and 156 spp. in the Neotropics (Øllgaard, 2012a, b) totaling 270 spp.

In the need of a revision of the system of Lycopodiaceae, Øllgaard (1987) proposed a new classification for the family and divided *Huperzia* s.l. into 22 groups, which he himself considered as informal and loosely defined.

In the Neotropics 12 of these groups are represented (Øllgaard, 1992). The *Huperzia selago* group consists of the genus *Huperzia s.str.* and the other 11 groups belong to *Phlegmariurus*. This classification were developed accordingly the growth habit (e.g., erect, ascending, recurved, pendulous); stem thickness; shoot differentiation (homoblastic or heteroblastic); leaf differentiation (homophyllous, heterophyllous, or gradually heterophyllous); leaf arrangement (number of leaves in whorls), crowding (distance between whorls); leaf outline and solid shape (e.g., thickness, vein prominence, margin curvature), margin shape (e.g., teeth, rugulate, smooth), leaf dimensions, direction, color, texture, surface (smooth, papillate), stomate distribution, shape of epidermal cells; shape and color of decurrent leaf bases; sporangium shape and size and spore size.

More recently, the classification system for Lycophytes and ferns presented by The Pteridophyte Phylogeny Group (PPG I, 2016). The Lycopodiaceae are treated into three subfamilies: Lycopodielloideae, Lycopodioideae, and Huperzioideae. This classification is an agreement based on the most recent molecular evidences of the monophyly of each lineage.

Lycopodielloideae is composed by four genera and ca. 54 species, distributed in the genera: *Lateristachys* Holub (four species), *Lycopodiella* (15 spp.), *Palhinhaea* (25 spp.), and *Pseudolycopodiella* (10 spp.).

Lycopodioideae is composed by nine genera and ca. 58 species, distributed in the genera: *Austrolycopodium* (eight species), *Dendrolycopodium* (four spp.), *Diphasiastrum* (20 spp.), *Diphasium* (five spp.), *Lycopodiastrum* (one sp.), *Lycopodium* (15 spp.), *Pseudodiphasium* (one sp.), *Pseudolycopodium* (one sp.), and *Spinulum* (three spp.).

Huperzioideae is composed by three genera and ca. 276 species, distributed in the genera: *Huperzia* (25 species), *Phlegmariurus* (250 spp.), and *Phylloglossum* (one sp.).

At the present proposal, we shall consider only one family Lycopodiaceae with three subfamilies (Lycopodielloideae, Lycopodioideae, and Huperzioideae) comprising 16 genera according to Wagner & Beitel (1992), Haines (2003), Øllgaard (2012a), Prado *et al.,* (2015), Field *et al.,* (2016), and PPG I (2016).

Brazilian Lycopodiaceae and its distribution

In Brazil, 52 species are known, circumscribed into nine genera: *Austrolycopodium*, *Diphasiastrum*, *Huperzia s.str., Diphasium, Lycopodium s.str.*, *Lycopodiella s.str.*, *Phlegmariurus*, *Palhinhaea*, and *Pseudolycopodiella* Holub (Prado *et al.*, 2015). An interesting fact is that there is only one record of a *Huperzia* species to Brazil, *H. catharinae* (Christ.) Holub, known only from the type collection made in 1890 (Øllgaard & Windisch, 1987, 2014).

Prado *et al.,* (2015) reported 40 species of *Phlegmariurus* to Brazil, which 24 are endemic and they are concentrated in the humid forests, which are located in the high mountains of Southeastern Brazil, in the area occupied by the Atlantic Rainforest and by the rocky fields (*campos rupestres*). The Amazon Forest is relatively low in representatives (Øllgaard & Windisch, 1987).

Endemism in the highlands (*campos de altitude*) and in the high Atlantic Forest seem to be clearly allopatric events followed by climate change, mountains lifting and the insular feature of the tops of the hills (inselbergs), with most endemic species in rocky fields probably arose from common ancestors (Alves & Kolbek, 1994, 2010; Safford, 2007). These regions are considered as one of the three primary centers of diversity of ferns and lycophytes of tropical America (Tryon, 1972). Despite the high dispersal ability of the spores of these groups, geographical isolation can be effective at distances within the dispersion range is especially favored by ecological isolation. Thus, allopatric speciation has been especially important in the development of the high number of endemic species, which characterize these areas (Tryon, 1972). Moreover, *Phlegmariurus* and *Huperzia* show very strong biogeographic patterns, perhaps related to their underground holosaprophytic gametophytes and aspects of its establishment (Wikström, 2001).

Previous phylogenetic studies

Wikström & Kenrick (1997, 2000a, 2001), Wikström *et al.,* (1999), and Ji *et al.,* (2008) had already published phylogeny studies including some *Phlegmariurus* species with several molecular markers (*rbc*L, *trn*L intron, *trn*L-F intergenic spacer, and ITS regions), however these studies did not sample significantly the species as a whole. The results of these analyses have shown that the groups proposed by Øllgaard (1987) did not form monophyletic groups and no current infrageneric classification was proposed. Besides, a Paleotropical and Neotropical species clades have formed two sister lineages, with two exceptions, probably results of long distances dispersal events (Wikström *et al.*, 1999).

Field *et al.* (2016) published a compreensive phylogeny for the family with 119 taxa, four chloroplast loci (*rbc*L, *trn*L intron, intergenic spacers *trn*L-F, *psb*A-*trn*H), and also the optimization of 29 morphological characters onto the phylogeny. Their findings show two major lineages that correspond to the subfamilies Lycopodioideae and Huperzioideae sensu Wagner & Beitel (1992). The lycopodioid clade includes subclades that may correspond to the genera *Lycopodium* and *Lycopodiella* with their respective subdivisions sensu Øllgaard (1987) or the several genera sensu Øllgaard (2012b). Their outcomes also indicate that a three-genus (*Phylloglossum*, *Huperzia* s.str., and *Phlegmariurus* s.l.) classification of huperzioid Lycopods are supported by molecular evidence and best reflects evolutionary, ecological, and morphological divergence within the lineage. For *Phlegmariurus*, they covered up to 68 species (about 25% of the total species number), but only 27 species from the Neotropics, with no new Brazilian accessions, since their focus was to better understand the generic relationships.

Burnard *et al.* (2016) examined the global relationships of all New Zealand's Lycopodiaceae species through only *rbc*L gene sequences. Against the current taxonomy proposals, they recognize only five genera in Lycopodiaceae: the Huperzioid triad *Phylloglossum*, *Huperzia,* and *Phlegmariurus*; *Lycopodium* (incorporating *Pseudodiphasium*, *Austrolycopodium*, *Diphasium*, and *Pseudolycopodium*), and *Lycopodiella* (incorporating *Pseudolycopodiella*, *Lateristachys*, and *Palhinhaea*). Besides, the resulting tree presented does not support the monophyly of *Lycopodium* and *Huperzia*. The phylogeny was also largely consistent with the suggested subdivision of *Lycopodiella* and *Lycopodium* into sections (Øllgaard 1987) or genera (Holub 1983, 1985; Øllgaard 2012) except by *Lycopodiella serpentina* (Kunze) B. Øllg. and *Lycopodiella carolininana* (L.) Pic.

Serm. that had been grouped into *Lycopodiella* sect. *Caroliniana* (Øllgaard 1987) or *Pseudolycopodiella* (Holub 1983) but were recovered as polyphyletic, suggesting a revision of these classification schemes. Furthermore, they were not able to distinguish the different morphologies and ecological variations of *Phlegmariurus varius* (R.Br.) A.R. Field & Bostock and *P. billardierei* (Spring) A.R. Field & Testo within the genetic analysis suggesting they constitute a single species. The PPG I (2016) revised this classification and accepted three subfamilies and the huperzioid lycopods were included in the subfamily Huperzioideae to reflect these findings.

In these studies (Wikström *et al.*, 1999; Field *et al.*, 2016), were sampled only *Phlegmariurus dichotomus* (Jacq.) W.H. Wagner, *Phlegmariurus linifolius* (L.) B. Øllg., *Phlegmariurus reflexus* (Lam.) B. Øllg., and *Phlegmariurus wilsonii* (Underw. & F.E. Lloyd) B. Øllg. that also occurs in Brazil, but they are collections from other countries. Even in Wikström *et al.* (1999), the authors stated that "Brazil is not represented in our analysis, but data from this area could be particularly important for clarifying the relationships of Neotropical and African species". Moreover, the relationship of the endemic species from Brazil and other species of the genus are still uncertain. Prado & Hirai (2014) discuss the importance of using molecular data to better understand the Brazilian endemic species, mainly because most of them are restricted to Atlantic Rainforest. Thus, a detailed molecular analysis, with emphasis on Brazilian species is necessary and will surely bring new data to understand the group as a whole.

Regarding the lack of Brazilian species samples in the phylogenetic analysis and highly endemism of the genus in Brazil, this study aims to (1) to perform a molecular phylogenetic analyses of the Brazilian species of *Phlegmariurus*, (2) to test whether the endemic species of *Phlegmariurus* of Brazil form one or more

monophyletic groups and (3) to optimize morphological and anatomical characters on the resulting trees in order to understand the evolution of those among the species.

Material and Methods

Fieldwork and Taxon Sampling

Species samples were collected in their natural habitat. During the fieldwork were made all the notes corresponding to the habit of the species, date of collection, habitat, collection site, as well as other information needed for ongoing research. The collection expeditions followed the indications of locations displayed in Øllgaard & Windisch (1987), data contained in herbaria visited, and based on the data present in online databases (e.g., INCT - Herbário Virtual). The botanical material was collected according to the usual techniques of collection, described by Radford *et al.* (1974). For the molecular biology study, the material was collected and preserved in silica gel, according to the methodology described by Chase & Hills (1991).

Ten field expeditions were realized to the following locations: Campos do Jordão, Paranapiacaba State Park, and Serra da Bocaina National Park, in São Paulo State. Itatiaia, Serra dos Órgãos, and Tijuca National Parks in Rio de Janeiro State. RPPN Serra do Caraça and Ibitipoca State Park in Minas Gerais State.

We collected 20 species of *Phlegmariurus*, 13 of them endemic to Brazil (54% of the total endemic species to the country) (Appendix 1). Some species of *Phlegmariurus* can be seen in Figures 1 and 2.

The specimens were deposit at SP Herbarium (Herbário Maria Eneyda P. Kauffman Fidalgo, Instituto de Botânica – São Paulo), and duplicates were distributed to SPF Herbarium of the University of São Paulo and AAU Herbarium of

the University of Aarhus, Denmark. Remaining duplicates were also sent to NY, RB, MBM, and other Brazilian Herbaria.

The internal group consisted of 96 new accessions of *Phlegmariurus* species (Appendix 1) along with the already available sequences in GenBank (Benson *et al.,* 2004) for the markers chosen here.

The outgroups were selected based on Field *et al.,* (2016) comprising species of the genera *Huperzia* and *Phylloglossum drummondii*, as well as Paleotropical species of *Phlegmariurus*. All these sequences were previously generated and were available at GenBank. The list of the sequences used can be consulted at the appendix 1.

Taxonomic studies in Herbaria

Several herbaria collections were consulted in order to learn about the species, their morphology, and geographical distribution, especially to target the best locations to realize the fieldwork. Additionally, during these visits some fragments were taken to be used in the molecular studies when no fresh material was found in the expeditions.

Seven herbarium collections were analyzed. The SP Herbarium (Instituto de Botânica de São Paulo), RB (Jardim Botânico do Rio de Janeiro), R (Museu Nacional), ESA (Escola Superior de Agricultura 'Luiz de Queiroz'), UEC (Universidade de Campinas), HRCB (UNESP – Rio Claro), and CESJ (Universidade de Juiz de Fora). As expected, the analysis of collections provided a guidance in collecting expeditions (with information that is not yet accessible in online databases), also assisted in species recognition in the field and the correct identification of specimens. The specimens analyzed contributed in the morphological

study of the species and some of the exceptional characters are presented below. As well, three species were sampled from herbarium material for the molecular analysis.

Morphological studies, as well as the identification of specimens collected were based on taxonomic works of the family (e.g., Nessel, 1955; Øllgaard & Windisch, 1987; Øllgaard, 1992; Wagner & Beitel, 1992; Haines, 2003; Pita *et al.*, 2006a, b; Ramos & Sylvestre, 2010; Arana & Øllgaard, 2012; Windisch & Øllgaard, 2014). These studies were used to define groups of species, as well as optimizing the clades with morphological characters that were evaluated after obtaining the molecular data and phylogenetic analyses.

Molecular markers selection

Three chloroplast markers were selected to be investigated: the *rbc*L gene (~ 1300 bp), the *trn*L intron (~ 700 bp), and the *trn*L-F intergenic spacer (~600 bp).

The chloroplast gene *rbc*L is widely studied in plant phylogenies to explore higher hierarchical levels as subfamilies, families, orders, and classes because they evolve at a lower speed (Gielly & Taberlet, 1994; Judd *et al.*, 2009). The *rbc*L may function well for establishing phylogenetic relationships in more specific levels of ferns (genera and species, e.g., Schneider *et al.*, 2002; Ranker *et al.*, 2003; Skog *et al.*, 2004; Ranker *et al.*, 2004; Prado *et al.*, 2013). However, in lycophytes, the *rbc*L has been more preserved, creating the need to combine analyses with more variable regions of the genome (Wikström & Kenrick, 2000a; Rydin & Wikström, 2002; Korall & Kenrick, 2004).

The intron *trn*L and the intergenic spacer *trn*L-F were chosen based on the good resolutions presented in previous studies with Lycopodiaceae or related groups (Wikström & Kenrick, 1999, 2000a), which allow consistency in the comparison of

results and access to gene sequences available in GenBank for these markers. Furthermore, recent studies have shown the effectiveness of the combination of *rbc*L and *trn*L intron as good loci for molecular identification of ferns (Groot *et al*., 2011; Li *et al*., 2011) and lycophytes (Wikström & Kenrick, 1999).

Molecular studies

The *rbc*L gene, the *trn*L intron, and *trn*L-F spacer region were amplified in one independent PCR for most samples. The PCR mixture was made to 20 μL, with the following concentration of reagents: 2.0 µl of USB® buffer (10x), 0.2 µl BSA (10mg/ml), 0.4 µl of dNTPs Qiagen® (10mM), 1.0 µl of forward primers (10µM), 1.0 µl of reverse primers (10µM), 0.3 µl of Taq polymerase USB® (5u/µl), and 1.5 µl from total genomic DNA.

Program details for *rbc*L marker include an initial denaturation (2 min at 95° C), then 35 cycles of denaturation (30 s at 95° C), annealing (30 s at 45° C), and extension (1.5 min at 72° C), then a final extension for 5 min at 72° C. For the *trn*L and *trn*L-F markers, initial denaturation (5 min at 94° C), then 35 cycles of denaturation (1.0 min at 94° C), annealing (30 s at 50° C), and extension (1.0 min at 72° C), then a final extension for 10 min at 72° C.

Amplified products were run on an agarose gel to visualize DNA concentrations and after were purified using ExoSAP-IT following the manufacturer's instructions (1 unit/µL; Affymetrix, Santa Clara, California, USA). Then, they were sequenced by Sanger Method on Human Genome and Stem-Cell Research Center (HUG-CELL) at University of São Paulo (USP). Primers used for sequencing reactions for *rbc*L were ESRBCL1F, ESRBCL628F, ESRBCL654R and

ESRBCL1361R (Schuettpelz & Pryer, 2007), for *trn*L, c (B49317) and e (B49873) and for *trn*L-F, d (A49855) and f (A50272) universal primers (Taberlet *et al*., 1991).

Phylogenetic Analyses

The resulting sequences were aligned and edited visually using Geneious 9.1 software (Kearse *et al*., 2012). The newly generated sequences were compared and aligned to available sequences from the GenBank aiming the more closely related groups based on previously published phylogenies (Wikström & Kenrick 1997, 2000a,b; Field *et al.*, 2016).

The 5' extremities of the sequences were excluded due to low informative data and a lot of missing data from the GenBank sequences. Trees were rooted using the outgroup method and the clade *Phylloglossum + Huperzia* were used, accordingly to Field *et al.* (2016).

Phylogenetic analyses were run using maximum-parsimony (MP), maximum-likelihood (ML), and Bayesian inference (BI) algorithms. The MP analyses were performed using the software PAUP v.4.0 beta10 for PC (Swofford, 2003) with a heuristic search with 1000 random addition sequence replicates, tree bisection-reconnection branch-swapping and 1,000,000 maxtrees.

Maximum likelihood analyses (ML) were conducted in RAxML-HPC program BlackBox (Stamatakis, 2014) and rounds the portal Cyberinfrastructure for Phylogenetic Research (CIPRES) v.3.3 (Miller *et al.*, 2010). For these tests, we used the algorithm implemented, which let you perform searches of the best tree and getting branches bootstrap support (BS) (Felsenstein, 1985) in a single analysis

(Stamatakis *et al.*, 2008). Thus, they were executed in 1000 BS replies with a subsequent search of the maximum likelihood tree, using nucleotide substitution model GTRGAMA + I, the rate category number values substitution of nucleotides (c) and the likelihood of acceptance level (-e) pattern (25 and 0.1, respectively).

The program MrBayes 3.1.6 (Huelsenbeck & Ronquist, 2001) was used for BI analyses and rounds the portal Cyberinfrastructure for Phylogenetic Research (CIPRES) v.3.3 (Miller *et al.*, 2010). The Bayesian analyses require evolutionary models, which were determined by using the program jModeltest 2.1.1 (Darriba *et al.*, 2012) for Linux. For *rbc*L, the evolutionary model was set to GTR + G (lsetnst = 2) with gamma distributed rate variation among sites (lset rates = gamma). For *trn*L, the evolutionary model was set to TMV + G (lsetnst = 6) with gamma distributed rate variation among sites (lset rates = gamma). For *trn*L-F, the evolutionary model was set to TVM + G (lsetnst = 6) with gamma distributed rate variation among variable sites (lset rates = invgamma). And for the concatenated matrix, the evolutionary model was set to GTR + G + I (lsetnst = 6) with gamma distributed rate variation among variable sites (lset rates = invgamma). The Bayesian phylogeny was estimated using two runs of four chains each, one cold, three hot, for 10,000,000 generations using the Markov Chain Monte Carlo (mcmc) search algorithm. Each 1000th generation was sampled. The analyses resulted in 10,000 trees with a convergence diagnostic value of <0.01. The first 2,500 trees were then discarded as the 25% burnin, and a 50% majority rule consensus tree computed from the remaining trees.

Results

Molecular analyses

Were obtained 31 new sequences from *rbc*L*,* 27 from *trn*L intron, and 27 from *trn*L-F intergenic spacer.

To perform the phylogenetic reconstruction, we run independent analyses (MP, BI, and ML) for each marker in addition to a combined one as follow. With the marker *rbc*L, the MP (Figure 3), the BI (Figure 4), and the ML (Figure 5) analyses showed the monophyly of *Phlegmariurus* (MP bootstrap = 100%, BI PP = 1.00, ML bootstrap = 97%) and a clade with the Brazilian species (Clade H) with high moderate and support (MP bootstrap = 75%, BI PP = 0.99, ML bootstrap = 72%).

With the marker *trn*L intron, the MP (Figure 6), the BI (Figure 7), and the ML (Figure 8) analyses also confirmed the monophyly of *Phlegmariurus* (MP bootstrap = 99%, BI PP = 1.00, ML bootstrap = 100%) and presented the clade H with high support (MP bootstrap = 90%, BI PP = 1.00, ML bootstrap = 98%).

With the marker *trn*L-F intergenic spacer, in the MP (Figure 9), the BI (Figure 10), and the ML (Figure 11) analyses, *Phlegmariurus* is monophyletic as well (MP bootstrap = 99%, BI PP = 1.00, ML bootstrap = 97%), the clade H is paraphyletic, still the inner clades (J and I) are monophyletic with high support (Clade J: MP bootstrap = 99%, BI PP = 1.00, ML bootstrap = 98%; Clade I: MP bootstrap = 91%, BI PP = 1.00, ML bootstrap = 93%).

The final combined matrix of the three plastid regions included 48 species of *Phlegmariurus* from the Neotropical region, seven species from the Paleotropical region including *Phlegmariurus phlegmaria* (L.) T. Sen & U. Sen, the type species of the genus, and three species of an outgroup formed by *Phylloglossum* plus *Huperzia*.

To perform the phylogenetic reconstruction we run independent analyses (MP, BI, and ML) for the combined analyses as follow (Figures 12, 13, 14, and 15).

The major lineages

The clade **A** is composed by the outgroup with *Phylloglossum drummondii* plus the species of *Huperzia*. The clade **B** is formed by some Paleotropical species of *Phlegmariurus* plus *P. funiformis*, a Neotropical species that appears at the Paleotropical clade. The clade **C** is composed exclusively by Neotropical species, but *P. ophioglossoides* which is from Tanzania.

The clade **D** is composed only of epiphytic species and the species *Phlegmariurus fontinaloides*, *P. ericifolius*, *P. subulatus*, *P. heteroclitus*, *P. ophioglossoides*, *P. erythrocaulon,* and *P. biformis* are all heterophyllous plants.

The clade **E** is formed by slender, flaccid pendulous species, that can be epiphytic or terrestrial pendulous. *Phlegmariurus tenuis*, *P. comans,* and *P. acerosus* refer to the Neotropical "*Huperzia verticillata* group". The collection *Gissi 107* is affine to *P. comans* but with more robust leaves and an erect habit. The exception in the clade **E** is *P. intermedius* that is a rupestral species very similar to *P. reflexus*. Indeed, *P. intermedius* is a slender homophyllous plant, generally crawling over grasses and rocks. The more delicate habit of the plants is common in this clade.

The clade **F** recovers the "*Huperzia dichotoma* group", except by *Phlegmariurus nudus*. Still, all these species presents filiform or linear leaves and mostly patent.

The clade **G** is formed by *Phlegmariurus lindenii* plus a group formed by the clades **H** and **K**. *Phlegmariurus lindenii* is a flaccidly pendulous species, with

often homophyllous shoots. The clade **H** is composed only of Brazilian species with two distinct clades, the clade **I** with rupestral species of the Brazilian highlands and the clade **K** with pendulous epiphytic species that grow in forests. **I** and **K** emerged monophyletic and with strong supports in all analyses made.

Phlegmariurus deminuens, *P. recurvifolius*, *P. pungentifolius,* and *P. regnellii* are rupestral species, growing erect from high elevations, especially in the state of Minas Gerais. They are very robust plants, with coriaceous leaves. The clade **I** presents strong support (MP bootstrap = 100%, ML bootstrap = 100%, BI PP = 1.00), but the relationships between the species are low supported or represent polytomies.

The clade **J** is formed by species characterized by their epiphytic habit and the differentiation of the leaves. Here we recovered the "*Huperzia heterocarpon* group" plus *Phlegmariurus quadrifariatus* and *P. hexastichus.*

The clade **L** may be considered a *Phlegmariurus reflexus* complex, with the species presenting reflexed leaves as their main characteristic. Interestingly, the three terminals identified as *P. reflexus* does not emerge as monophyletic, possibly indicating different species under the same name.

The Clade **M** recovers *Phlegmariurus christii* and *P. hemleri*. These species are very similar morphologically by the presence of denticulate ascending leaves, both from Southeastern Brazil on banks at high altitudes. *Phlegmariurus hemleri* differs from *P. christii* by having broader and larger leaves in more spaced whorl, meanwhile, in *P. christii,* the leaves are more congested.

The clade **N** is composed of Andean terrestrial species with often with

characteristically fingerlike shoots.

Besides the two exceptions (*Phlegmariurus funiformis* (Cham. ex Spring) B. Øllg. and *P. ophioglossoides* (Lam.) A.R. Field & Bostock) also presented in other studies, all Neotropical species has risen as a monophyletic group with strong support (MP bootstrap = 98%, fig. 12; BI PP = 1.00, fig. 13; ML bootstrap = 98%, fig. 14?), but the relations within the four major lineages (shown as **D**, **E**, **F**, and **G** groups) stood up with different topologies in different analyses. In MP (Figure 12), the groups are presented as ((**D E**) **F G**), in BI (Figure 13) as ((**F** (**D E**)) (**G**)), while in ML (Figure 14) as (**D** (**E** (**F** (**G**)))), hitherto it is hard to tell the most adequate relationship.

In the group **G**, *Phlegmariurus lindenii* (Spring) B. Øllg. rises as a sister group from the rest of the species in all analyses (*P. lindenii* (**H** and **K**)) with high support (MP bootstrap = 94%, BI PP = 1.00, ML bootstrap = 100%). Brazilian species, (endemics) are found spread in all groups, not forming a monophyletic group aswhole, but some species are intimate related, especially the **H** group formed only with Brazilian species (MP bootstrap = 99%, BI PP = 1.00, ML bootstrap = 100%,), mostly endemics, and with two clades inside (**I** and **J**). The clade **I** is represented by the terrestrial species (MP bootstrap = 100%, BI PP = 1.00, ML bootstrap = 100%) from Brazilian highlands and the clade **J** contain the epiphytes species (MP bootstrap = 98%, BI PP = 1.00, ML bootstrap = 100%). The **K** group (MP bootstrap = 50%, ML bootstrap = 67%, BI PP = 0.81) is composed mostly by terrestrial species from the Andes and some from Brazil.

The results from the separated markers trees were low informative (Table 1). Thus only the results from the combined dataset using MP, ML, and BI analyses were discussed (Figure 15).

Table 1. Details concerning the alignments used in the present study.

	*rbc*L	*trn*L	*trn*L-F	Combined
Total number of terminals	48	58	57	67
Included characters	1263	624	620	2507
Variable characters	142	168	200	510
Parcimony-informative characters	76	87	99	262

Figure 1. Species of epyphytic *Phlegmariurus*. **A.** *P. fontinaloides* (Gissi *et al., 153*) **B.** *P. heterocarpon* (Gissi *et al., 160*) **C.** *P. biformis* (Gissi *et al., 159*) **D.** *P. acerosus* (Prado & Hirai, *2349*). **A**, **B,** and **C** were collected at Bocaina National Park and **D** at Campos de Jordão [D. T. Vasques (**A**) and R. Hirai (**B. C. D.**)]

Figure 2. Terrestrial species of *Phlegmariurus* **A.** *P. ruber* (Gissi & Colletta, *169*) **B.** *P. pungentifolius* (Gissi & Colletta, *172*) **C.** *P. nudus* and *P. comans* growing side by side (Gissi *et al., 106*) **D.** *P. regnellii* (Williams, *469*) **E.** *Phlegmariurus reflexus* with ascending microphylls (Gissi *et al., 273*) **F.** *P. reflexus* with reflexed microphylls (Gissi *et al., 165*). **A** and **B** were collected at Serra do Caraça; **C** at Itatiaia National Park; **D** at Serra de Caldas; **E** and **F** at Bocaina National Park [C. M. Mynssen (**D**)].

Figure 3. Strict consensus tree from Maximum parcimony analysis of chloroplast *rbc*L sequences. MP bootstrap at the nodes.

Figure 4. Strict consensus tree from Bayesian analysis of chloroplast *rbc*L sequences. Posterior probabilities at the nodes.

Phylloglossum drummondii

63 *Huperzia selago*

Huperzia serrata

98 P. verticilatus

P. petiolatus

P. phlegmaria

P. mingcheensis

P. squarrosus

P. hippuris

P. erythrocaulon

P. biformis Gissi 159

P. biformis Gissi 129

P. heteroclitus

P. fontinaloides

74 P. mollicomus

P. flexibilis

P. acerosus

P. comans Gissi 107

99 P. intermedius

P. comans Gissi 100

P. nudus

P. wilsonii

P. mandiocanus

P. hippurideus

100

I P. deminuens Gissi 230

P. deminuens Gissi 229

100 P. pungentifolius

P. recurvifolius

P. regnellii

H P. silveirae

90 P. heterocarpon Prado 2348

J P. heterocarpon Gissi 131

97 P. martii

P. quadrifariatus

P. loefgrenianus Prado 2342

P. loefgrenianus Prado 2343

P. hexastichus

B R A Z I L

P. campianus

P. reflexus Gissi 233

P. reflexus Gissi 155

P. reflexus Gissi 165

85 P. reflexus Sundue 3089

P. linifolius

P. cummingii

P. attenuatus

P. christii Gissi 109

P. hemleri

67 P. christii Gissi 110

0.007

Figure 5. Strict consensus tree from Maximum likelihood analysis of chloroplast *rbc*L sequences. ML bootstrap at the nodes.
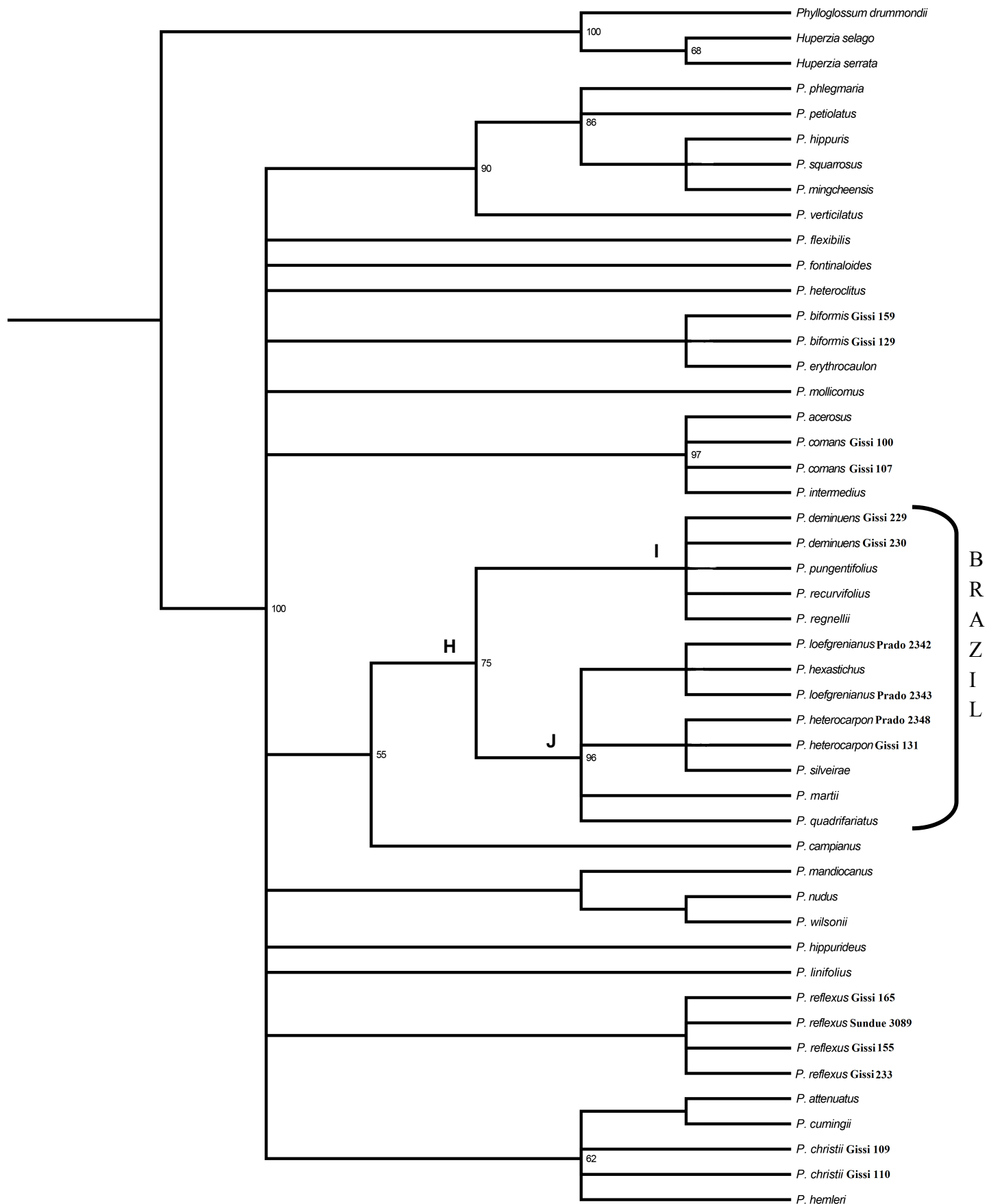
Figure 6. Strict consensus tree from Maximum parcimony analysis of chloroplast *trn*L sequences. MP bootstrap at the nodes.

Figure 7. Strict consensus tree from Bayesian analysis of chloroplast *trn*L sequences. Posterior probabilities at the nodes.

Phylloglossum drummondii

Huperzia selago

100

99 *P. verticilatus*
    P. funiformis
    P. phlegmaria
    P. squarrosus

100

99 *P. flexibilis*
    P. fontinaloides
    P. ericifolius
    P. subulatus
    P. heteroclitus
    P. biformis **Gissi 129**
    P. ophioglossoides
    P. erythrocaulon

100 *P. tenuis*
     P. intermedius
     P. comans **Gissi 107**
     P. comans **Gissi 100**
     P. acerosus

84

75 *P. dichotomus*
    P. mandiocanus
    P. wilsonii
    P. hippurideus
    P. nudus

H **I**
98   91 *P. pungentifolius*
           P. regnellii
           P. recurvifolius
           P. deminuens **Gissi 229**
           P. deminuens **Gissi 230**
       J *P. hexastichus*
       95 *P. loefgrenianus* **Prado 2342**
           P. loefgrenianus **Prado 2343**
           P. quadrifariatus
           P. heterocarpon **Prado 2348**
           P. heterocarpon **Gissi 131**
           P. silveirae

99

B
R
A
Z
I
L

P. lindenii

P. campianus
P. sarmentosus
P. linifolius
P. rosenstockianus

63

P. eversus
P. reflexus **Øllgaard 100617**
P. unguiculatus
P. reflexus **Gissi 233**
P. reflexus **Gissi 165**
P. attenuatus
P. tetragonus
P. crassus
P. capellae
P. polydactylus
P. rufescens
P. hystrix
P. compactus
P. cumingii
P. christii **Gissi 109**
P. hemleri
P. christii **Gissi 110**

0.02

Figure 8. Strict consensus tree from Maximum likelihood analysis of chloroplast *trn*L sequences. ML bootstrap at the nodes.

Figure 9. Strict consensus tree from Maximum parcimony analysis of chloroplast *trn*L-F sequences. MP bootstrap at the nodes.
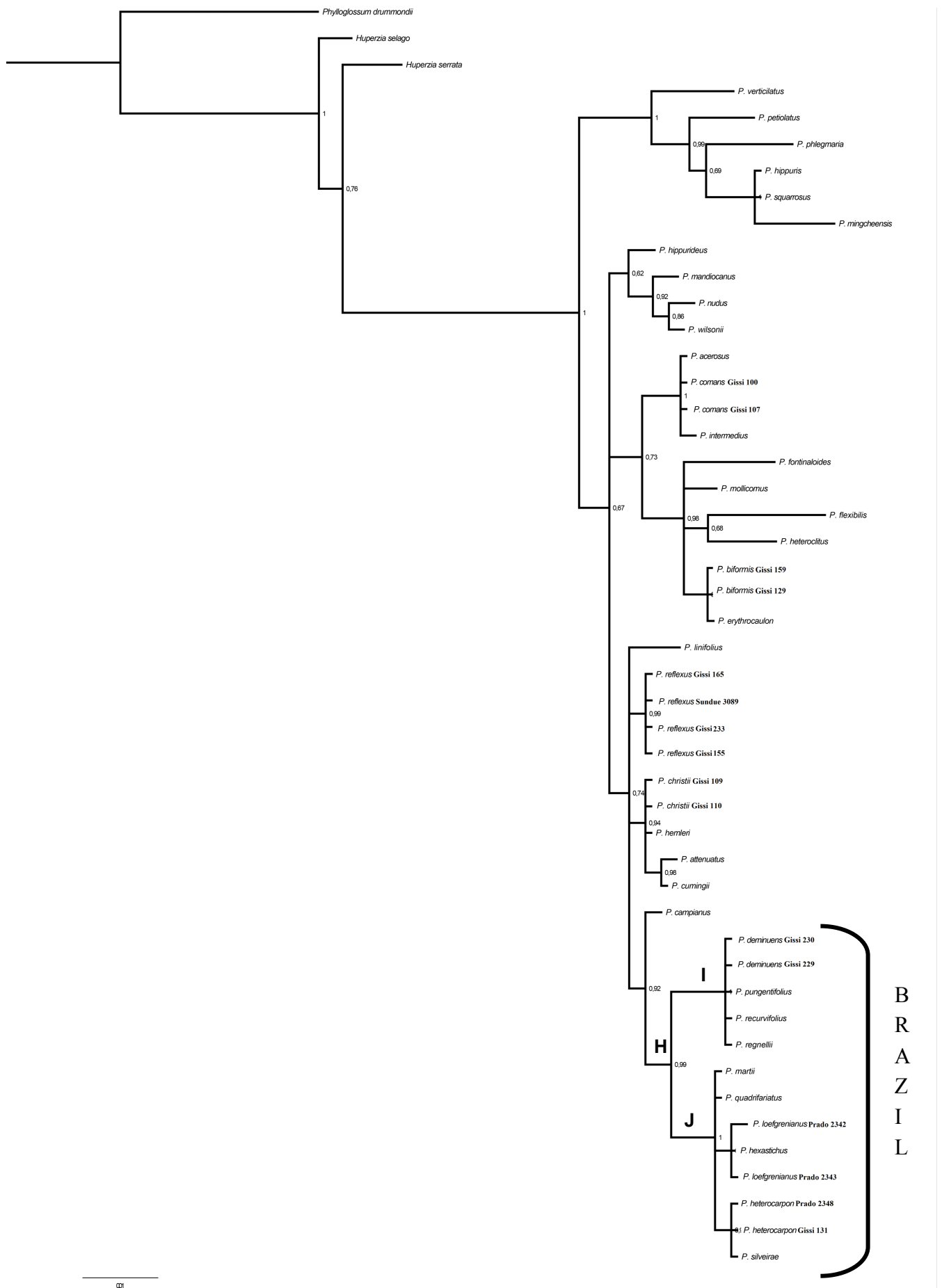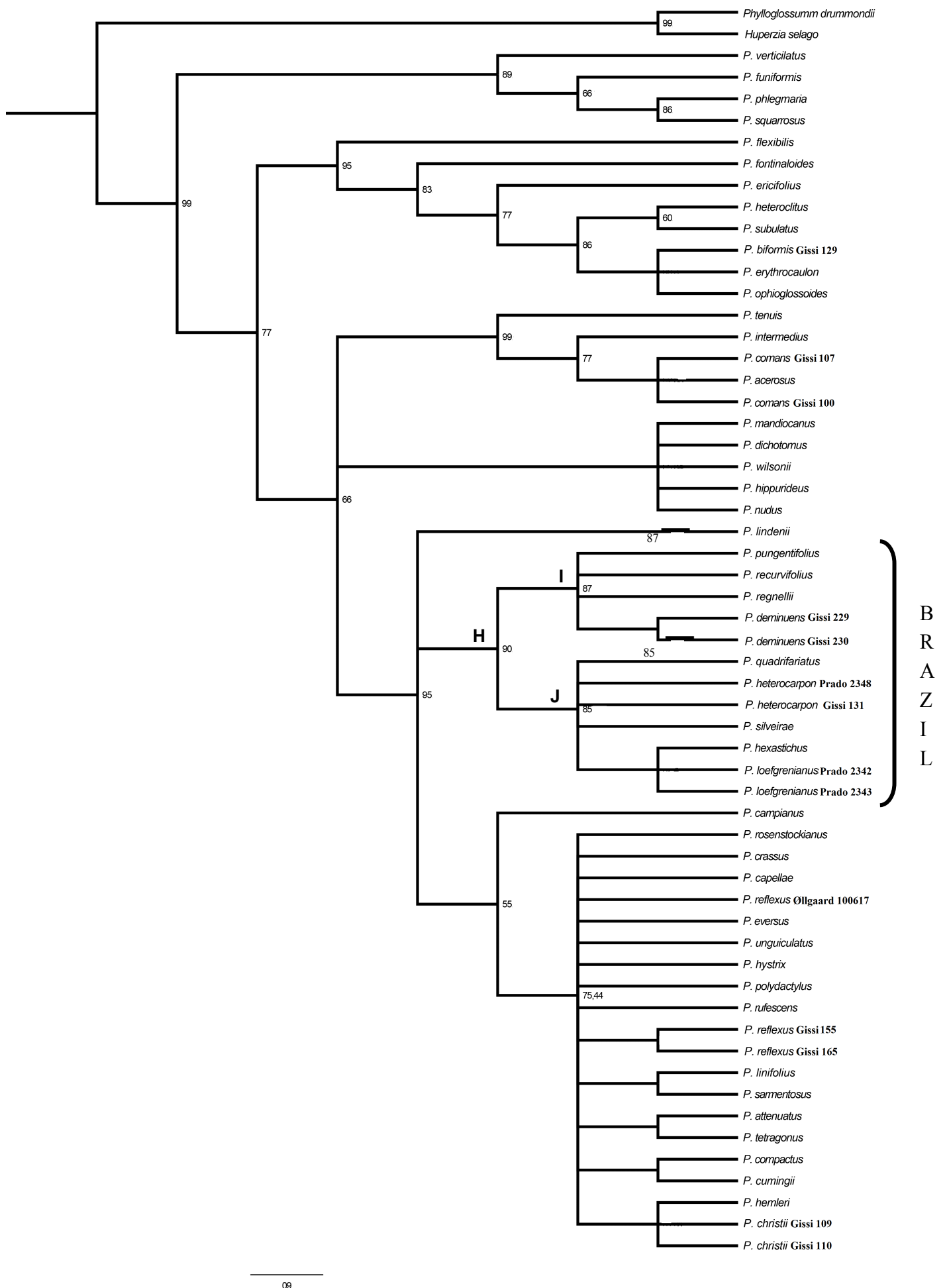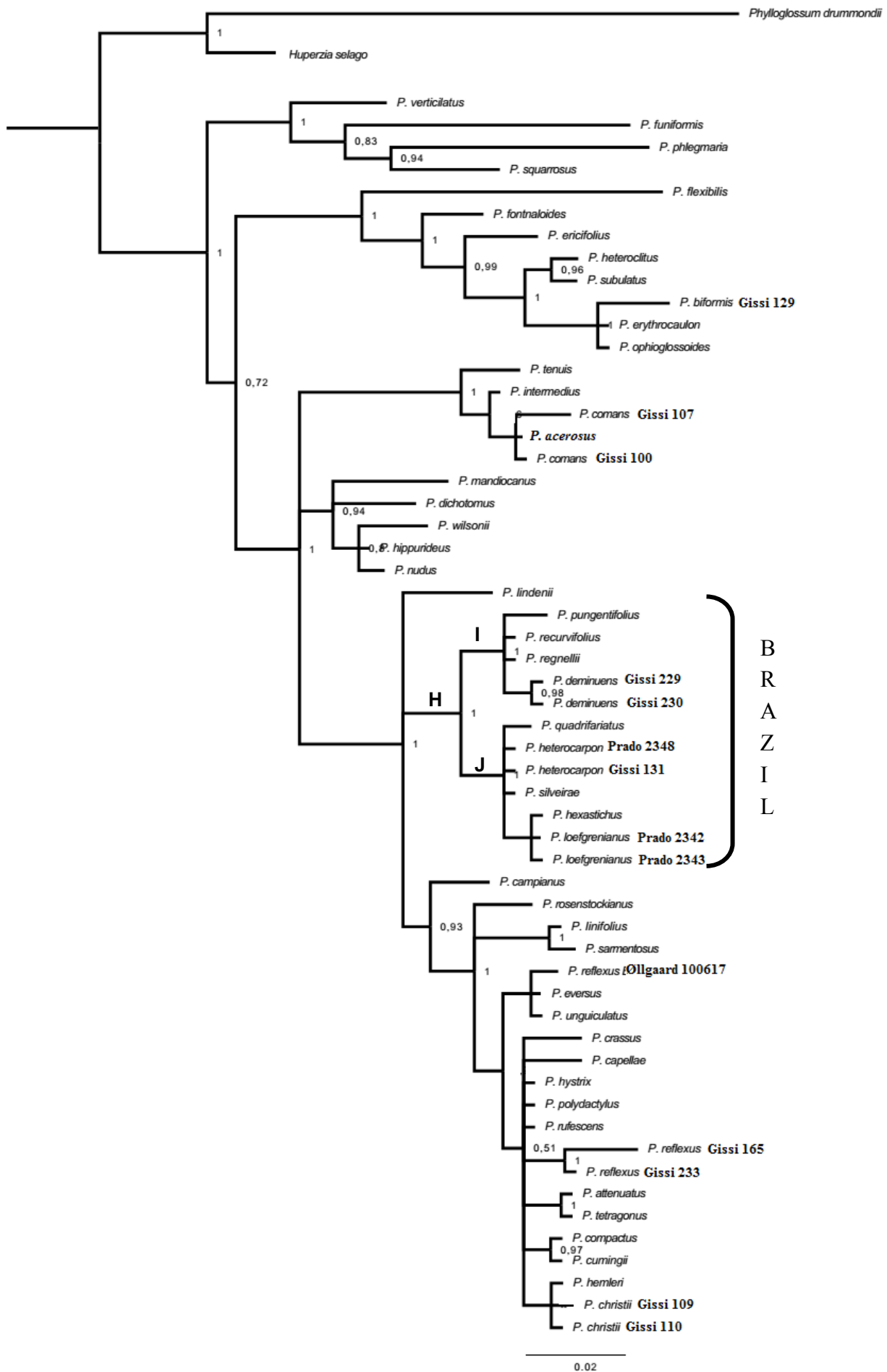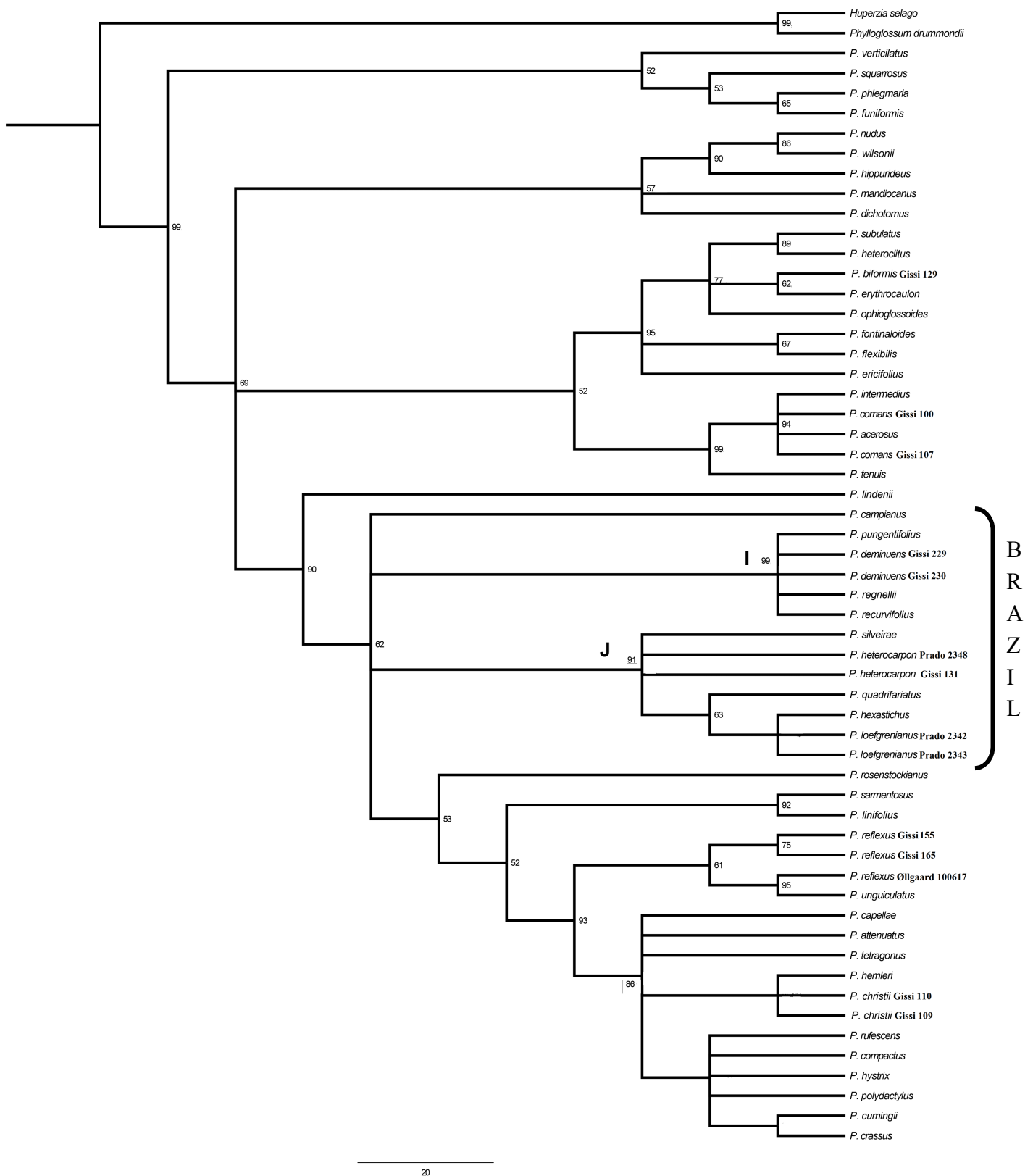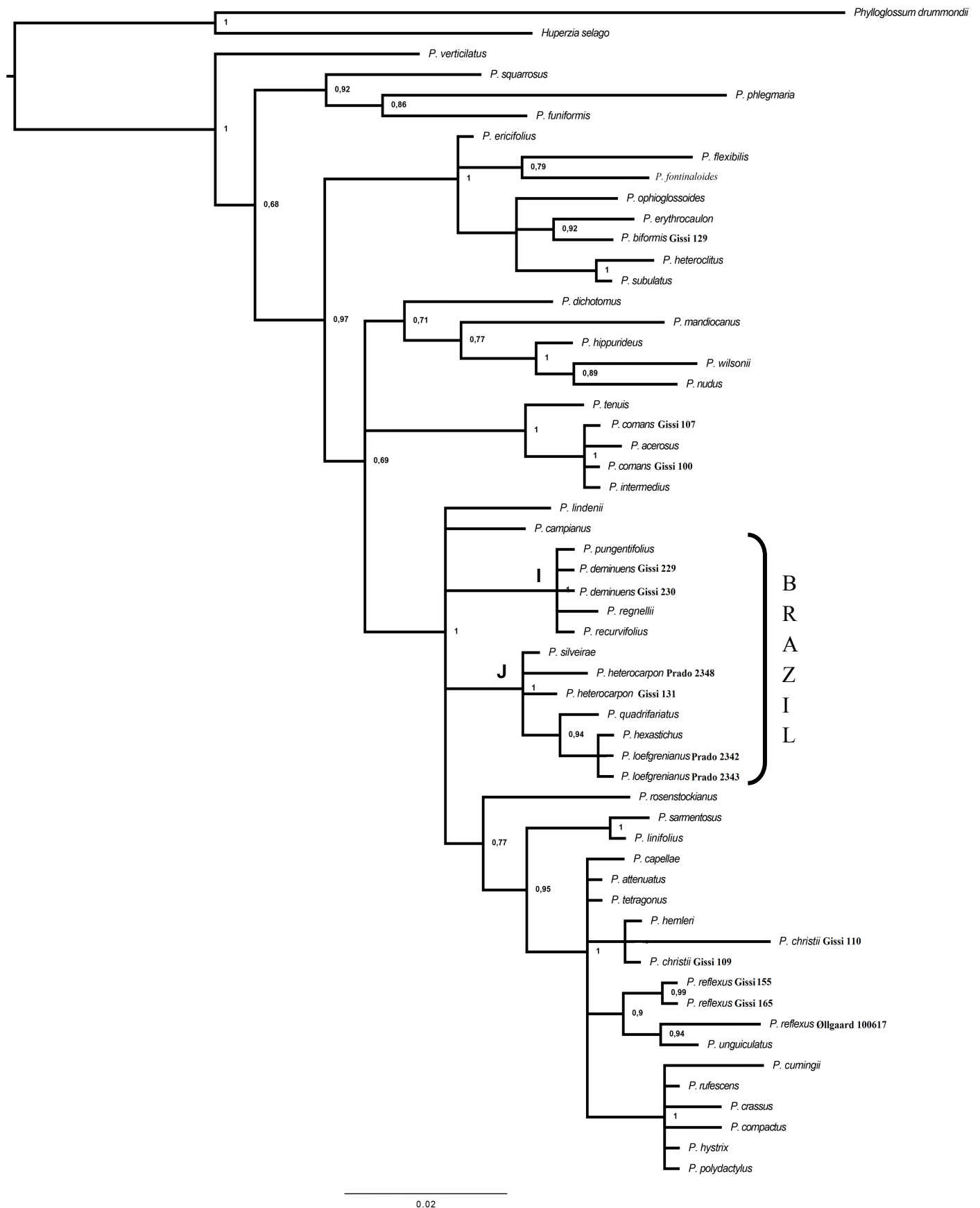
Figure 10. Strict consensus tree from Bayesian analysis of chloroplast *trn*L-F sequences. Posterior probabilities at the nodes.

Figure 11. Strict consensus tree from Maximum likelihood analysis of chloroplast *trn*L-F sequences. ML bootstrap at the nodes.

Figure 12. Strict consensus tree from Maximum parcimony analysis of chloroplast *rbc*L, *trn*L and *trn*L-F sequences. MP bootstrap at the nodes.

Figure 13. Strict consensus tree from Bayesian analysis of chloroplast *rbc*L, *trn*L and *trn*L-F sequences. Posterior probability at the nodes.
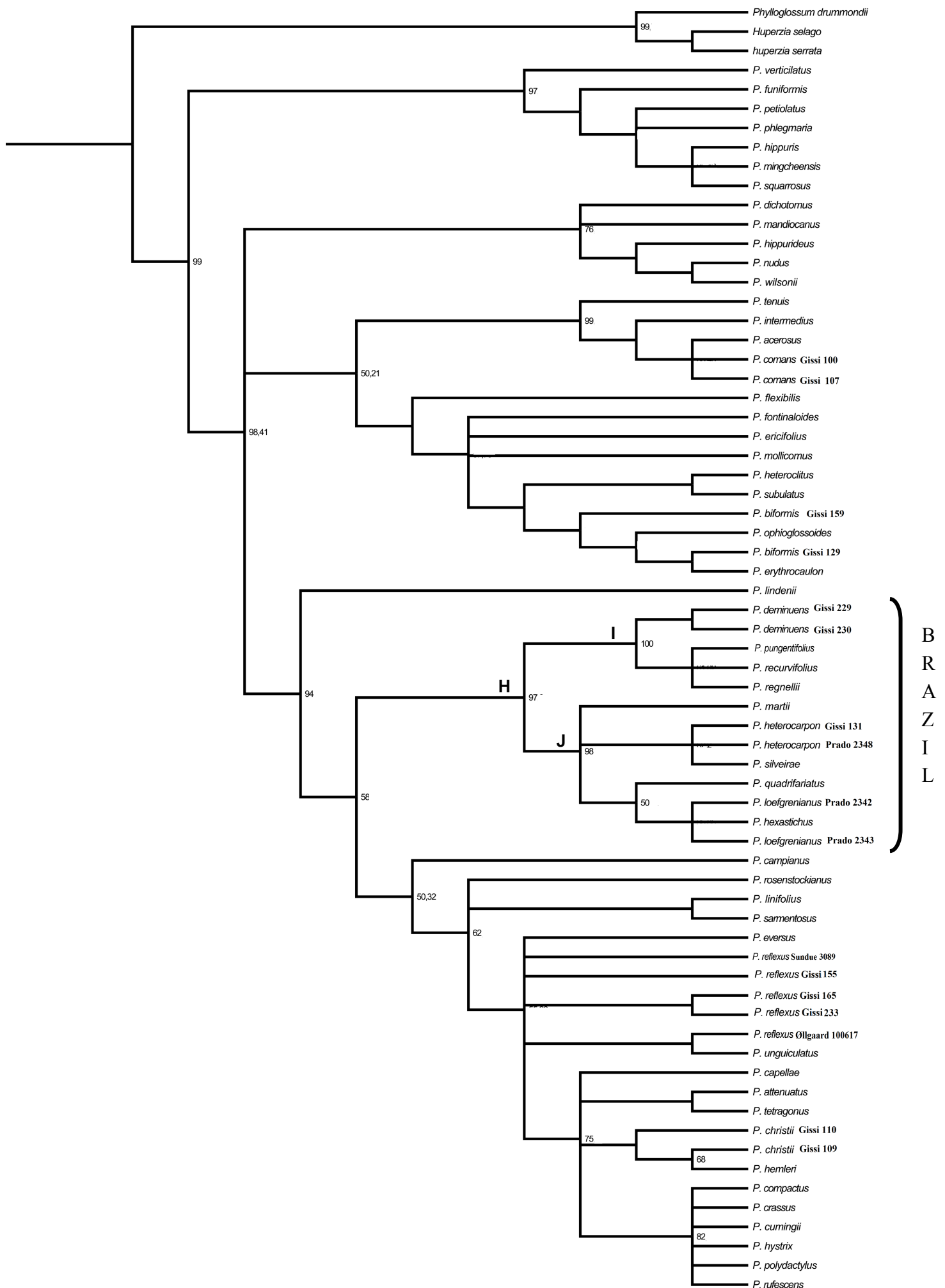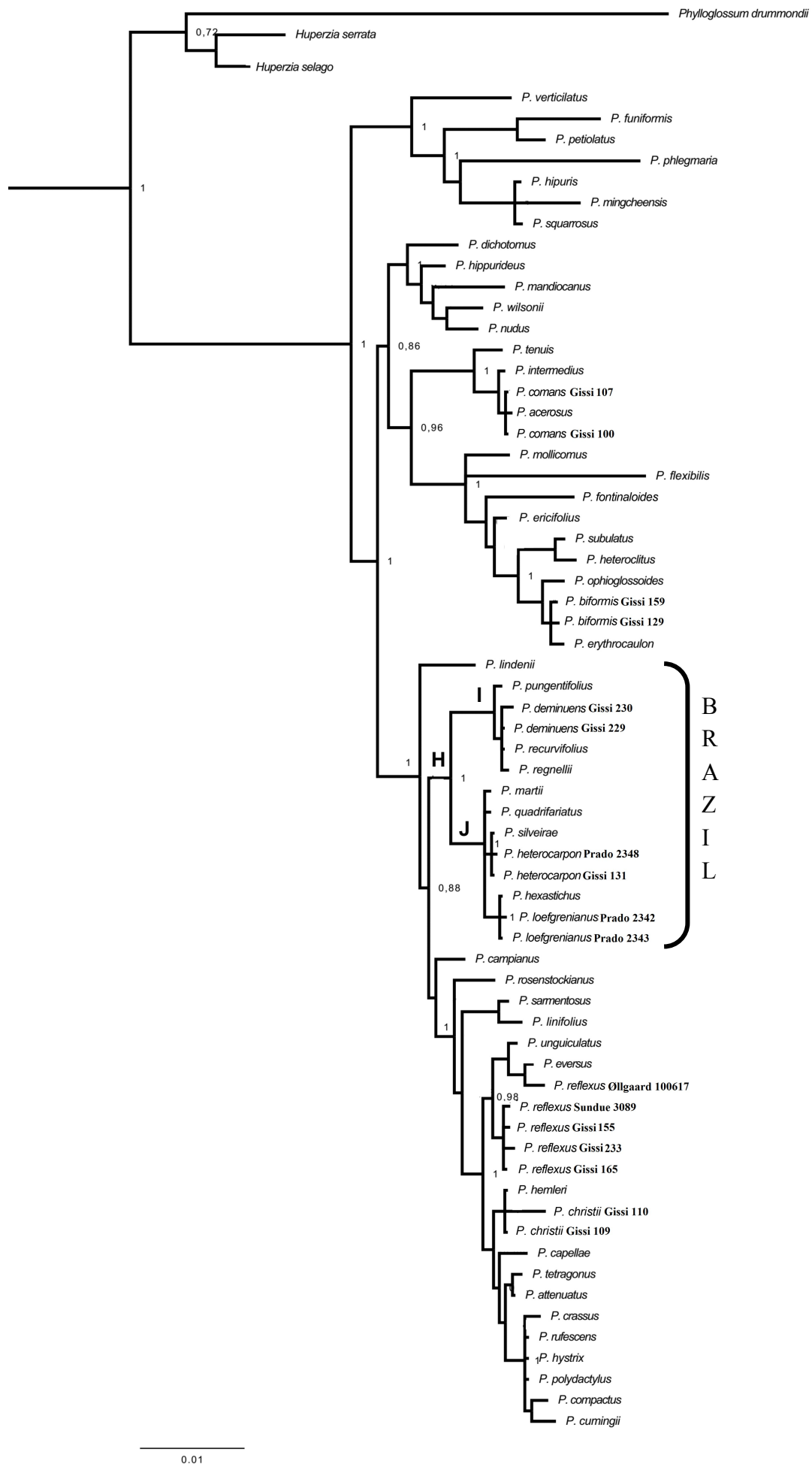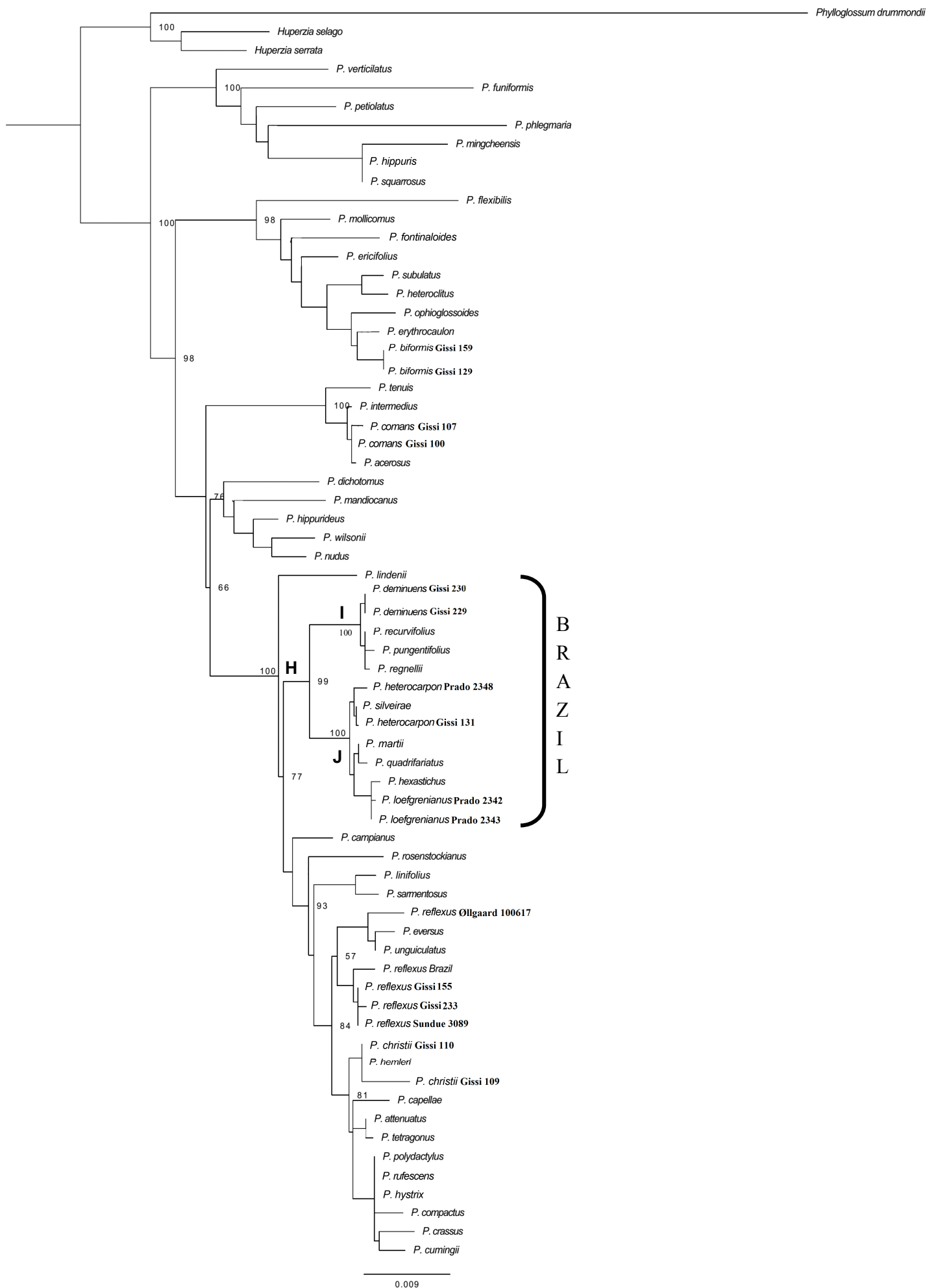
Figure 14. Strict consensus tree from Maximum likelihood analysis of chloroplast *rbc*L, *trn*L and *trn*L-F sequences. ML bootstrap at the nodes.

Outgroup

Paleotropics

Neotropics

Brazil

Endemics ★

Node	Label
A	99/100/0.72 — *Phylloglossum drummondii*, *Huperzia selago*, *Huperzia serrata*

Phylloglossum drummondii
Huperzia selago
Huperzia serrata

A 99/100/0.72

B 97/100/1.00
Phlegmariurus verticillatus
Phlegmariurus funiformis
Phlegmariurus petiolatus
Phlegmariurus phlegmaria
Phlegmariurus mingcheensis
Phlegmariurus hippuris
Phlegmariurus squarrosus

D 97/98/1.00
Phlegmariurus flexibilis ★
Phlegmariurus mollicomus
Phlegmariurus fontinaloides ★
Phlegmariurus ericifolius
Phlegmariurus subulatus
Phlegmariurus heteroclitus
Phlegmariurus ophioglossoides
Phlegmariurus erythrocaulon ★
Phlegmariurus biformis Gissi 129 ★
Phlegmariurus biformis Gissi 159 ★

E 99/100/1.00
Phlegmariurus tenuis
Phlegmariurus intermedius
Phlegmariurus comans Gissi 129 ★
Phlegmariurus comans Gissi 100 ★
Phlegmariurus acerosus

F 76/89/1.00
Phlegmariurus dichotomus
Phlegmariurus mandiocanus
Phlegmariurus hippurideus
Phlegmariurus wilsonii
Phlegmariurus nudus ★

C 98/100/1.00

Phlegmariurus lindenii

I 100/100/1.00
Phlegmariurus deminuens Gissi 229 ★
Phlegmariurus deminuens Gissi 230 ★
Phlegmariurus recurvifolius
Phlegmariurus pungentifolius ★
Phlegmariurus regnellii ★

H 99/100/1.00

J 98/100/1.00
Phlegmariurus heterocarpon Prado 2348
Phlegmariurus heterocarpon Gissi 131
Phlegmariurus silveirae ★
Phlegmariurus martii ★
Phlegmariurus quadrifariatus ★
Phlegmariurus hexastichus ★
Phlegmariurus loefgrenianus Prado 2343 ★
Phlegmariurus loefgrenianus Prado 2342 ★

G 94/100/1.00

Phlegmariurus campianus
Phlegmariurus rosenstockianus
Phlegmariurus linifolius
Phlegmariurus sarmentosus

K 50/67/0.81

L ?/57/0.98
Phlegmariurus reflexus Øllgaard 100617
Phlegmariurus eversus
Phlegmariurus unguiculatus
Phlegmariurus reflexus Brazil
Phlegmariurus reflexus Gissi 165
Phlegmariurus reflexus Gissi 233
Phlegmariurus reflexus Sundue 3089

M 94/97/1.00
Phlegmariurus christii Gissi 109 ★
Phlegmariurus christii Gissi 110 ★
Phlegmariurus hemleri ★

N ?/58/0.58
Phlegmariurus capellae
Phlegmariurus attenuatus
Phlegmariurus tetragonus
Phlegmariurus polydactylus
Phlegmariurus rufescens
Phlegmariurus hystrix
Phlegmariurus compactus
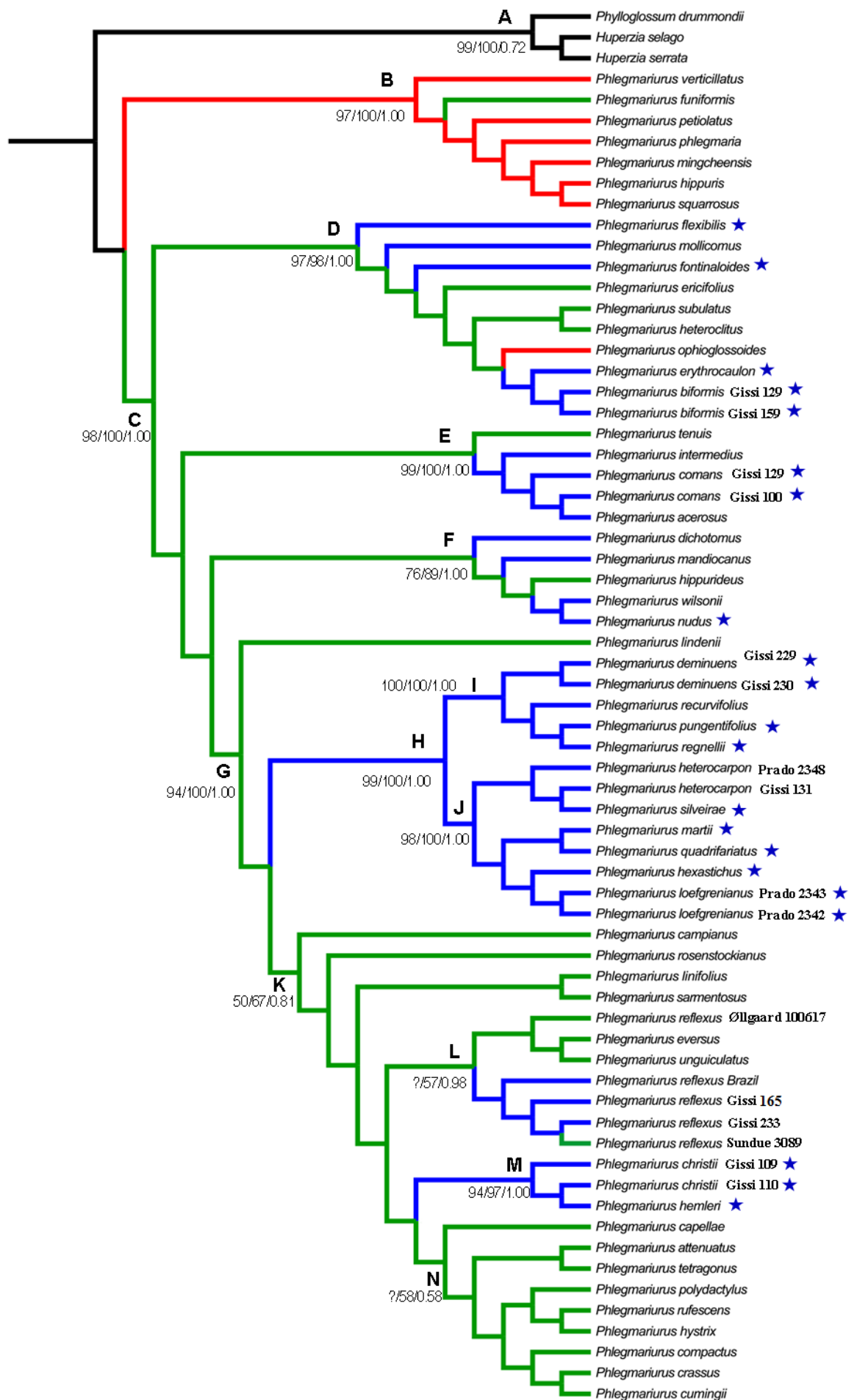*Phlegmariurus crassus*
Phlegmariurus cumingii

Figure 15. Maximum likelihood phylogeny of *Phlegmariurus* samples from analysis of chloroplast *rbc*L, *trn*L and *trn*L-F sequences. Nodes mentioned in the text are lettered A–N and support values (maximum parsimony bootstrap, maximum likelihood bootstrap, and Bayesian posterior probability. ? = different topology in MP) are shown for internal nodes. Tree with real length of branches in the left side. Paleotropical species in red, Neotropical species in green, and Brazilian species in blue. Brazilian endemic species are indicated with a star.

35

Discussion

Based on these results, it is possible to state the monophyly of the subfamily Huperzioideae composed of the genera *Phylloglossum*, *Huperzia,* and *Phlegmariurus*, corroborating the tree genera proposal (PPG I, 2016). In all analyses, *Phlegmariurus* form a monophyletic group (MP bootstrap = 100%, BI PP = 1.00, ML bootstrap = 100%,) with two clades: one with the Neotropical and another with the Paleotropical species, as shown in previous phylogenetic analyses with the group (Wikström & Kenrick, 1997; Wikström *et al.*, 1999; Field *et al.*, 2016, Burnard *et al.*, 2016).

Several distinct lineages appeared within the genus (Figure 15). The Paleotropical species (Clade **B**) form a distintic lineage with Neotropical species (Clade **C**). Within the Neotropical clade, several groups are formed, including all Brazilian species indicating that they evolved after the continental split from Africa.

Brazilian *Phlegmariurus* species diversified alongside of extra-Brazilian species, mainly occurring in the Andes. Wikström & Kenrick (2001) estimated the divergence times in Lycopodiaceae by use of nonparametric rate smoothing and nucleotide sequences of the *rbc*L gene. They concluded that much extant species diversity in Lycopodiaceae stems from relatively recent cladogenic events and the coalescence of the recent Neotropical *Phlegmariurus* species dates to 79 Million years.

One hypothesis for this disjunction of the species is several long distance dispersal events between the Atlantic Rainforest and the Andes, especially for spore dispersal plants (Muñoz *et al.,* 2004; Kyrkjeeide *et al.,* 2016). Other hypothesis is that from an ancient link between the Andes and Atlantic Rainforest that favored

migration of the species with a later retraction of the forest isolating the populations (Calvente *et al.,* 2011).

A link between the Brazilian Páramos (campos de altitude) and Andean highland vegetation has been documented (Safford, 2007), suggesting a potential migration route through northern Argentina or the Paraguayan lowlands, the highlands of Uruguay, and Southern Brazil.

The Brazilian endemic species of *Phlegmariurus* do not form one monophyletic group. However, one group (Clade **H**) of endemics form a monophyletic group highly supported in all analysis, and within this group two lineages are represented, the clade **I** and **J**. The clade **I** formed by *P. deminuens*, *P. recurvifolius*, *P. pungentifolius,* and *P. regnellii*. These species are morphological related and perhaps these species have evolved recently especially by their endemic characteristic, occurring mainly in the highlands of the state Minas Gerais. The clade **J** formed by *P. heterocarpon*, *P. silveirae*, *P. martii*, *P. quadrifariatus*, *P. hexastichus*, and *P. loefgrenianus*. They represent the "*Huperzia heterocarpon* group" (Øllgaard, 1992) plus *P. quadrifariatus* and *P. hexastichus*. Øllgaard (1987) describes this group with monomorphic leaves or gradually to abruptly dimorphic, and in some species decussate in terminal divisions. He also comments that "some species are perhaps related to *H. quadrifariata*". The leaves differentiation and the epiphytic habit are possible synapomorphies of the group, but the relations among the species are poor solved.

Another lineage with interesting characteristics is the clade **L**, that we may call "The *Phlegmariurus reflexus* complex". *Phlegmariurus reflexus* is recognized by the reflexed denticulate leaves, but in the revision for the Neotropics, Øllgaard (1992) states that *P. reflexus* is a polymorphic species poorly understood,

yet there are several names as heterotypic synonyms of *P. reflexus* that may represent valid taxa, but their delimitation and distribution are still uncertain. *Phlegmariurus eversus* and *P. unguiculatus* are very similar to *P. reflexus* being distinguished by slight differences. *Phlegmariurus reflexus* collection *Øllgaard 100617* from Ecuador appeared in a different clade than the other collections from Brazil and the collection *Sundue 3089* from Mexico. The collection *Gissi 233* is affine to *P. reflexus* but differs by presenting margins of the leaves entire and a more robust habit. This voucher was collected in Ibitipoca State Park and there are many specimens with the same morphology, from the same locality, that appear undetermined in some visited herbaria. *Gissi 155* was collected at the Bocaina National Park, but in contrast to the other species in this clade, it presents ascending, abaxially convex leaves with entire margins. Those collections were thought to be different species but arose among *P. reflexus* terminals. Still, they may represent either new species, reestablishment of some taxa or only a morphological variation of the same species. Further studies of morphology, phylogeny and population genetics are necessary for better understanding the correct application of these names and crucial for the conservation status of these species.

The clade **M** is formed by *Phlegmariurus christii* and *P. hemleri*, that are very alike species even hard to distinguish in the field. Both are endemic species, homophyllous and terrestrial from high elevations. *Phlegmariurus rostrifolius* (Silveira) B. Øllg. is a poorly known high-altitude species, known only from the type collection from Serra do Papagaio, Minas Gerais, and perhaps not distinct from the *P. christii*. All these names may represent a unique species and a review for this group is necessary.

The clade **N** is clearly distinct composed by Andean terrestrial species. In these species, the leaves usually have a basal air cavity abaxially in the mesophyll, appearing as a bulge externally, and with shoots slightly to strongly heteroblastic, differentiated into erect sporangium-bearing shoots, and ascending to prostrate or subterranean and rhizomelike, rooting, rejuvenating shoots which continually give off erect shoots by equal dichotomies. The species of this clade were included in the "*Huperzia saururus* and *brevifolia* group" (Øllgaard, 1992). Still few represented in the molecular studies, these groups represent the high diversity encountered in the Ecuadorian and Colombian Páramos.

The phylogeny does not corroborate the previous classification by Øllgaard (1987) and Nessel (1955). The diversification of the group is more complicated than we expected and the morphological aspects used on those classifications emerged several times in the life story of *Phlegmariurus*. The leaf differentiation and the habit are the mainly characters used for the classification in the genus treatment (G. Herter 1949; Øllgaard 1987, 1992). Other morphological characteristics analyzed as leaf margins, orientation, color, and texture, were too variable among the clades and are low phylogenetic informative. Hitherto, with the lack of more sequences of Neotropical species, mainly the Andean's and more data about the morphology of the group as a whole, infrageneric classifications are still considered precocious.

Epiphytism and terrestrialization in Phlegmariurus

Another characteristic used especially for identification is the growth habit. *Phelgmariurus* can be composed of distinct habits ranging from erect terrestrial plants to pendulous epiphytes. Wikström *et al.,* (1999), in the second phylogenetic study of *Phlegmariurus*, studied the epiphytism and terrestrialization in the genus and documented a single origin of epiphytism and multiple reversals to a terrestrial habit. The epiphytism in *Phlegmariurus* probably arisen at Late Cretaceous along the diversification of the Angiosperms as presented in Wikström & Kenrick (2001). This pattern is widely observed in epiphytic ferns (Sundue *et al.*, 2015). By field observation, some pendulous species were found not only on top of trees but also on the ground relying on other plants or pending between rocks and ravines indicating a plasticity of habits some species can have.

Since the study of Wikström *et al.,* (1999), the epiphytism in Lycopodiaceae has been unraveling. Aside ferns and orchids, club mosses constitute one important group of vascular epiphytes in tropical forests. Since the soil contact is lost, epiphytes suffer a high risk of desiccation and need the capacity to quickly and directly use rainwater or moisture (Sundue *et al.*, 2015).

According to their tolerance level to drought Benzing (1990) divided the vascular epiphytes into three categories: hygrophytes, mesophytes, and xerophytes. Hygrophytes are strictly adapted to hygrophilous habitats and are drought intolerant. Owing to this drought intolerance, hygrophytes are observed only in places constantly submitted to abundant rains, such as the tropical mountain nebulous forests, also characterized by the highest diversity in epiphytes. Hygrophytes often display anatomy and morphology regressive traits facilitating direct water absorption.

Mesophytic epiphytes do not exhibit particular adaptive features but grow in places where water is easily available, such as in hygrophilous forests. They are thus often observed sympatrically with hygrophytes, or they are able to create a suspended soil and are thus called humus-collectors, displaying growth habits allowing them to accumulate humus and to entrap besides nutrients and water. Xerophytes display adaptive traits allowing them to avoid and/or endure drought. Xerophilous strategies (i.e., adaptations for limiting water loss – sclerophylly – and/or for storing water and nutrients – succulence –, in order to survive the drought period) are more widespread in epiphytic flowering plants. Xeromorphic features are also observed in numerous ferns as stout blades covered by a thick cuticle, waxes, stout suberified stems storing water and amylaceous reserves and water storage tissue.

The plants of *Phlegmariurus* may be considered hygrophytes, since the epiphytic species do not show humus collection strategies, neither xeromorphic features as mentioned above. On the other hand, the sporophytes of these plants feature corticular roots to develop a localized tree-anchoring root mass that probably facilitated the conquest of the epiphytic habit.

Pita *et al.,* (2006b) examined the external and internal morphology of the vegetative leaves of several species of *Phlegmariurus* and observed the presence of epidermal cells with sinuous anticlinal walls, invaginations in the inner side of the external periclinal wall of the epidermal cells and amphistomatic leaves. These anatomic features are related to scarce water environments as the above the trees or the top of the hills.

Nevertheless, not all *Phlegmariurus* are epiphytes, and as shown at the phylogeny (Figure 16), a reversal to a plesiomorphic terrestrial condition occurred

many times in the evolution of the Genus. The terrestrial species occur on the highlands and may have shown up related with the South American mountains orogeny.

As seen on previously phylogenies (Wikström *et al.*, 1999; Field *et al.*, 2016), reversal to the terrestrial habit occurred twice, but in this work, we can see two more reversals (Figure 16). Since some plants can be encountered as epiphytes, pendulous at ravines or among rocks, all poorly nourished environments. We suppose that these species can be transitional between epiphytes or pendoulous terrestrial conditions. This especially occurs in the species from the clades **E** and **F**, as *P. mandiocanus*, *P. wilsonii*, *P. dichotomus*, and *P. comans*. Truly terrestrial species are found in the clades **I** and **L**, **M**, **N**. These species are adapted to living in high elevate environments.

Leaf differentiation

Among the morphological characters observed during the study, we can highlight the leaf differentiation. Homophylly and heterophylly regard to leaf reduction in the apex of the plants, especially the sporophylls. The homophyllous plants present all the microphylls uniform, even the fertile ones. The gradually heterophyllous plants have a gradual reduction of the leaves and the heterophyllous have an abrupt reduction in contrast to the others. Øllgaard (1987) also called secondary homophylly when all the leaves are reduced and apressed as *P. quadrifariatus* (Bory) B. Øllg. and *P. hexastichus* (B. Øllg. & P. G. Windisch) B. Øllg., and expanded leaves are rare and found only at the base of the plant. Although

Øllgaard (1987) related secondary homophylly to neoteny from heterophyllous ancestors, no phylogenetic analysis have been done until now for unraveling this.

In our analysis, we observed species with heterophylly in many terminals of the Paleotropical and Neotropical species, including Andean and Atlantic Rainforest species (Figure 17?). Some degree of heterophylly (gradually heterophyllous, heterophyllous or secondary homophyllous) appeared twice among the Neotropical species at the clade **D** and **J**. Hence, we can consider two states of character for leaf differentiation that is either completely homophyllous during the whole sporophyte lifetime or presenting some sort of heterophylly, especially during the sporangia formation.

Figure 16. Optimization of the epiphytism and terrestrialization onto the Maximum likelihood phylogeny of *Phlegmariurus* samples from analysis of chloroplast *rbc*L, *trn*L and *trn*L-F sequences. Epiphytic species are marked in green while terrestrial species are in black. Brazilian endemic species are indicated with a star.
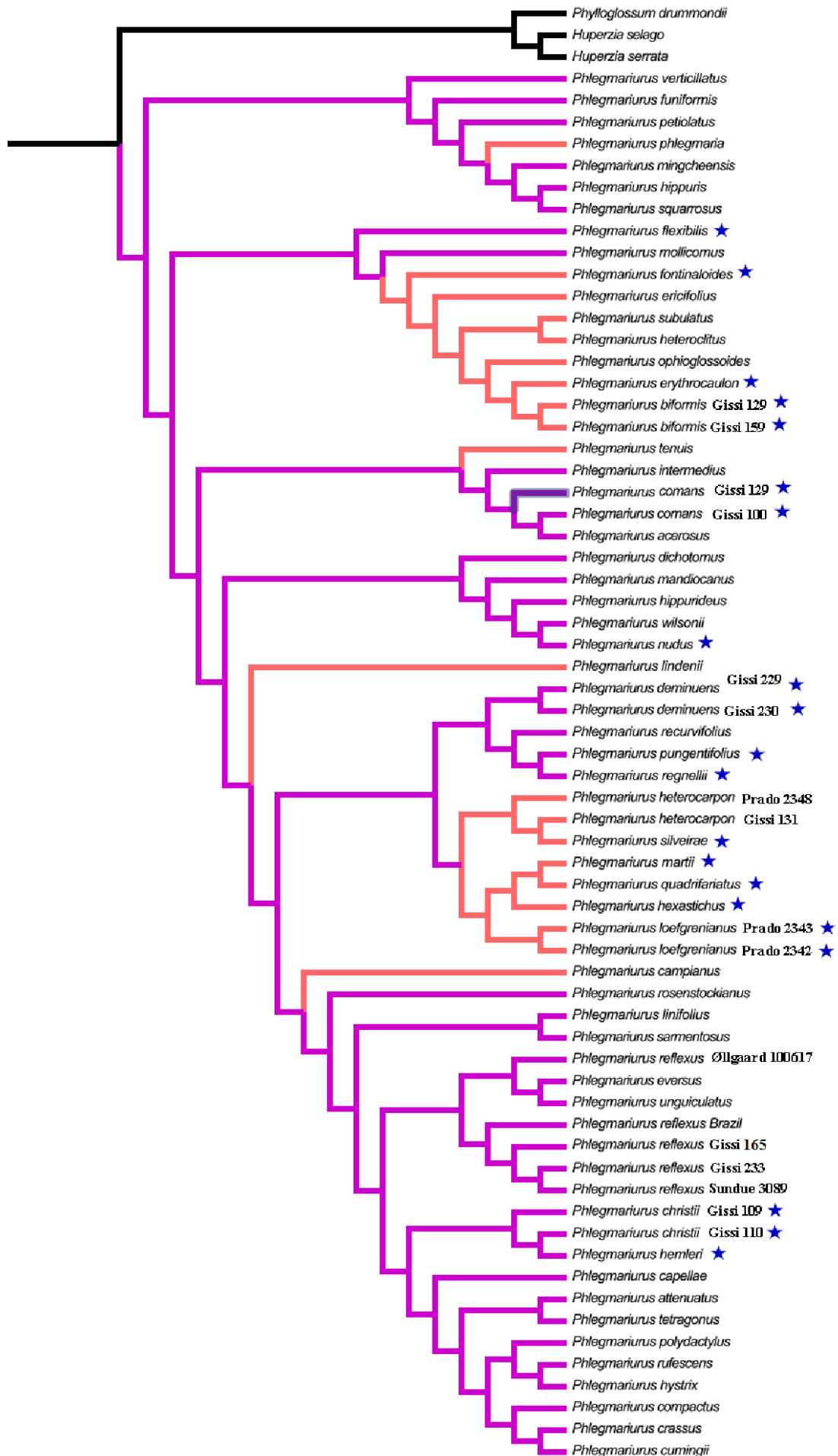
Figure 17. Optimization of the heterophylly onto the Maximum likelihood phylogeny of *Phlegmariurus* samples from analysis of chloroplast *rbc*L, *trn*L and *trn*L-F sequences. Heterophyllous species are marked in orange while homophyllous species are in black. Brazilian endemic species are indicated with a star.

Final Considerations

1. All Brazilian species of *Phlegmariurus* sampled aroused in the Neotropical clade merged with the Andean species.

2. The clade **H** form an important group of Brazilian endemic species with high support.

3. The epiphytism is considered plesiomorphic in the genus and terrestrialization appeared more than twice.

4. The groups suggested by Øllgaard (1987) are not congruent to the phylogeny, but some are supported with modifications.

5. Creating a new infrageneric classification is still presumptuous, whereas a broader sample is required and characters used in the current classifications do not rise as synapomorphies for many groups. Sequencing more molecular markers for more species would be interesting for better understand the relationships of the species in the smaller clades.

Resumo

Phlegmariurus é o gênero mais abundante de Lycopodiaceae no Brasil com 40 espécies, sendo 24 destas endêmicas, ocorrendo principalmente na Mata Atlântica e Campos Rupestres da Cadeia do Espinhaço. Alguns trabalhos de filogenia com o grupo já haviam sido realizados, contudo sem incluir uma amostragem representativa das espécies brasileiras. Dessa forma, realizamos a filogenia molecular das espécies de *Phlegmariurus* endêmicas do Brasil utilizando três marcadores moleculares de cpDNA. *Phlegmariurus* se mostrou monofilético, incluindo dois clados, um Neotropical e outro Paleotropical. Todas as espécies brasileiras amostradas apareceram no clado neotropical. As espécies endêmicas apareceram em vários clados em meio as espécies andinas. O epifitismo e a diferenciação dos microfilos foram otimizados nos clados obtidos e indicaram que essas características surgiram várias vezes na história evolutiva do grupo.

Abstract

Phlegmariurus is the most abundant genus of Lycopodiaceae in Brazil with 40 species, 24 of which are endemic, occurring mainly in the Atlantic Rainforest and Rocky Fields ("Campos Rupestres") in the Espinhaço range. Some phylogeny works with the group had already been carried out, but not including a representative sample of the Brazilian species. Thus, we performed a molecular phylogeny of *Phlegmariurus* species endemic in Brazil using three molecular markers of cpDNA. *Phlegmariurus* showed to be monophyletic, including two clades, one Neotropical and another Paleotropical. All the Brazilian species sampled appeared in the Neotropical clade. The endemic species appeared in several clades alongside Andean species. Epiphytism and the microphylls differentiation were optimized at the obtained clade and they appeared several times in the evolutionary history of the group.

References

ALVES, R.J.V. & KOLBEK, J. 1994. Plant species endemism in savanna vegetation on table mountains (Campo Rupestre). **Brazilian Vegetation 113**: 125–139.

ALVES, R.J.V., & KOLBEK, J. 2010. Can campo rupestre vegetation be floristically delimited based on vascular plant genera? **Plant Ecology 207**: 67–79.

ARANA, M.D. & ØLLGAARD, B. 2012. Revisión de las Lycopodiaceae (Embryopsida, Lycopodiidae) de Argentina y Uruguay. **Darwiniana 50**: 266–295.

BIRRI, M. A., FRANCO, M.A., VALLEJO, M.G., CARRO-JUÁREZ, M., & AGNESE, A.M. 2014. *Huperzia saururus* (Lam.) Trevis. (Lycopodiaceae) facilitates ejaculation in spinal cord transected male rats. **Journal of Ethnopharmacology 157**: 38–44.

BENSON, D.A., CAVANAUGH, M., CLARK, K., KARSCH-MIZRACHI, I., LIPMAN, D.J., OSTELL, J., & SAYERS, E.W. 2013. GenBank. **Nucleic Acids Research 41**(D1): D36–D42.

BENZING, D.H. 1990. Vascular Epiphytes Cambridge University Press. New York, 147p.

BERNHARDI, J.J. 1801. Tentamen alterum filices in genera redigendi. **Journal für die Botanik. [Edited by H. A. Schrader]. 1800** (2): 126.

BURNARD, D., SHEPHERD, L., PERRIE, L., & MUNKACSI, A. 2016. Phylogenetic relationships of New Zealand Lycopodiaceae. **Plant Systematics and Evolution 302**: 661–667.

CALVENTE, A., ZAPPI, D.C., FOREST, F., & LOHMANN, L.G. 2011. Molecular phylogeny, evolution, and biogeography of South American epiphytic cacti.

International Journal of Plant Sciences 172: 902-914.

CHASE, M.W. & HILLS, H.H. 1991. Silica gel: An ideal material for field preservation of leaf samples for DNA studies. **Taxon 40**: 215–220.

CHRISTENHUSZ, M.J.M., ZHANG, X.–C. & SCHNEIDER, H. 2011. A linear sequence of extant families and genera of lycophytes and ferns. **Phytotaxa 19**: 7–54.

DARRIBA, D., TABOADA, G.L., DOALLO, R., & POSADA, D. 2012. jModelTest 2: more models, new heuristics and parallel computing. **Nature Methods 9**: 772–772.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. **Evolution 39**: 783–791.

FIELD, A.R. & BOSTOCK, P.D. 2013. New and existing combinations in Palaeotropical *Phlegmariurus* (Lycopodiaceae) and lectotypification of the type species *Phlegmariurus phlegmaria* (L.) T. Sen & U. Sen. **PhytoKeys 20**: 33–51.

FIELD, A.R., TESTO, W., BOSTOCK, P.D., HOLTUM, J.A.M. & WAYCOTT, M. 2016. Molecular phylogenetics and the morphology of the Lycopodiaceae subfamily Huperzioideae supports three genera: *Huperzia*, *Phlegmariurus* and *Phylloglossum*. **Molecular Phylogenetics and Evolution 94**: 635–657.

GIELLY, L. & TABERLET, P. 1994. The use of chloroplast DNA to resolve plant phylogenies: noncoding versus *rbc*L sequences. **Molecular Biology and evolution 11**: 769–777.

GROOT, G.A., DURING, H.J., MAAS, J.W., SCHNEIDER, H., VOGEL, J.C. & ERKENS, R.H.J. 2011. Use of *rbcL* and *trnL–F* as a two–locus DNA barcode for identification of NW–European ferns: An ecological perspective. **PLoS**

ONE 6: e16371.

HAINES, A. 2003. **The families Huperziaceae and Lycopodiaceae in New England**. V.F. Thomas Co., Bowdoin. 100p.

HERTER, W. 1909. Beiträge zur Kenntnis der Gattung Lycopodium. Studien über die Untergattung *Urostachys*. **Botanische Jahrbücher Beiblätter 98**: 1–56.

HERTER, W. 1923. Itinera Herteriana III. Heteropteridophyta austroamericana. **Beihefte zum Botanischen Centralblatt 39**: 249.

HERTER, G. 1949. Index Lycopodiorum. Montevideo. I–IV, 1–120.

HERTER, G. 1950. Systema Lycopodiorum. **Revista Sudamericana de Botánica 8**: 67–86, 93–116.

HNATYSZYN, O., MOSCATELLI, V., GARCIA, J., RONDINA, R., COSTA, M., ARRANZ, C., BALASZCZUK, A., FERRARO, G. & COUSSIO, J.D. 2003. Argentinian plant extracts with relaxant effect on the smooth muscle of the corpus cavernosum of guinea pig. **Phytomedicine 10:** 669–674.

HOLUB, J. 1964. *Lycopodiella*, novy rod radu Lycopodiales. **Preslia 36**: 16–22.

HOLUB, J. 1983. Validation of generic names in Lycopodiaceae: with a description of a new genus *Pseudolycopodiella*. **Folia Geobotanica Phytotaxonomica 18**: 439–442.

HOLUB, J. 1985. Transfers of *Lycopodium* species to *Huperzia*: with a note on generic classification in Huperziaceae. **Folia Geobotanica Phytotaxonomica 20**: 67–80.

HUELSENBECK, J.L. & RONQUIST, F., 2001. MrBayes: Bayesian inference of phylogenetic trees. **Bioinformatics 17**: 745–755.

INCT – Herbário Virtual da Flora e dos Fungos. *http://inct.splink.org.br/*. Acesso em 13 de fevereiro de 2013.

JI, S.G., HUO, K.K., WANG J. & PAN, S.L. 2008. A molecular phylogenetic study of Huperziaceae based on chloroplast *rbcL* and *psbA–trnH* sequences. **Journal of Systematics and Evolution 46**: 213–219.

JUDD, W.S., CAMPBELL, C.S., KELLOGG, E., STEVENS, P. & DONOGHUE, M. 2009. **Sistemática Vegetal: Um enfoque filogenético.** 3ª. ed. (trad.). Artmed, Porto Alegre. 632p.

KEARSE, M., MOIR, R., WILSON, A., STONES–HAVAS, S., CHEUNG, M., STURROCK, S., BUXTON, S., COOPER, A., MARKOWITZ, S., DURAN, C., THIERER, T., ASHTON, B., MENTJIES, P. & DRUMMOND, A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. **Bioinformatics 28**: 1647–1649.

KORALL, P., KENRICK, P. & THERRIEN, J. 1999. Phylogeny of Selaginellaceae: evaluation of generic/subgeneric relationships based on *rbcL* gene sequences. **International Journal of Plant Sciences 160**: 585–594.

KORALL, P. & KENRICK, P. 2004. The phylogenetic history of Selaginellaceae based on DNA sequences from the plastid and nucleus: Extreme substitution rates and rate heterogeneity. **Molecular Phylogenetics and Evolution 31**: 852–864.

KYRKJEEIDE, M.O., HASSEL, K., FLATBERG, K.I., SHAW, A.J., BROCHMANN, C., & STENØIEN, H.K. 2016. Long-distance dispersal and barriers shape genetic structure of peatmosses (*Sphagnum*) across the Northern Hemisphere. **Journal of Biogeography 43:** 1215–1226.

LEHTONEN, S. & CHRISTENHUSZ, M.J.M. 2010. Historical herbarium specimens in plant molecular systematics – an example from the fern genus *Lindsaea*

(Lindsaeaceae). **Biologia 65**: 204–208.

LI, F–W, KUO, L–Y, ROTHFELS, C.J., EBIHARA, A., CHIOU, W–L, WINDHAM, M.D. & PRYER, K.M. 2011. *rbcL* and *matK* earn two thumbs up as the core DNA barcode for ferns. **PLoS ONE 6**: e26597.

MILLER, M.A., PFEIFFER, W., & SCHWARTZ, T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In **Proceedings of the Gateway Computing Environments Workshop** (GCE), 14 Nov. 2010, New Orleans, LA: 1 – 8.

MUÑOZ, J., FELICÍSIMO, Á.M., CABEZAS, F., BURGAZ, A.R., & MARTÍNEZ, I. 2004. Wind as a long-distance dispersal vehicle in the Southern Hemisphere. **Science 304**: 1144–1147.

MURPHY, R.A. & SARPONG, R. 2014. Heathcock–inspired strategies for the synthesis of fawcettimine–type *Lycopodium* alkaloids. **Chemistry–A European Journal 20**: 42–56.

NESSEL, H. 1955. Licopodiaceae. In: F.C. HOENE (ed.), **Flora Brasilica 2**: 1–131.

OLAFSDÓTTIR, E.S., HALLDORSDOTTIR, E.S., PICH, N.M. & OMARSDOTTIR, S. 2013. *Lycopodium* Alkaloids: Pharmacology: 1239–1262. In: K.G. Ramawat & J–M. Mérillon (eds.), **Natural Products – Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes**. Springer Verlag, Berlin.

ØLLGAARD, B. 1987. A revised classification of the Lycopodiaceae s. l. **Opera Botanica 92**: 153–178.

ØLLGAARD, B. 1992. Neotropical Lycopodiaceae – an overview. **Annals of the Missouri Botanical Garden 79**: 687–717.

ØLLGAARD, B. 1994. Lycopodiaceae. In: R.M. Tryon & R.G. Stolze (eds.),

Pteridophyta of Peru. Part VI: Marsileaceae–Isoetaceae. **Fieldiana, Botany, n.s. 34**: 16–66.

ØLLGAARD, B. 2012a. Nomenclatural changes in Brazilian Lycopodiaceae. **Rodriguésia 63:** 479–482.

ØLLGAARD, B. 2012b. New combinations in Neotropical Lycopodiaceae. **Phytotaxa 57**: 10–22.

ØLLGAARD, B. & WINDISCH, P.G. 1987. Sinopse das Lycopodiáceas do Brasil. **Bradea 5**: 1–43.

PITA, P.B., MENEZES, N.L. & PRADO, J. 2006a. Morfologia interna e externa de raiz e caule de espécies de *Huperzia* Bernh. (Lycopodiaceae – Pteridophyta) do Brasil. **Hoehnea 33**: 495–510.

PITA, P.B., MENEZES, N.L. & PRADO, J. 2006b. Morfologia externa e interna das folhas vegetativas, esporofilos e esporângios de espécies de *Huperzia* Bernh. (Lycopodiaceae – Pteridophyta) do Brasil. **Revista Brasileira de Botânica 29**: 115–131.

PPG I. 2016. A community-derived classification for extant lycophytes and ferns. **Journal of Systematics and Evolution 54**: 563–603.

PRADO, J., SCHUETTPELZ, E., HIRAI, R.Y. & SMITH, A.R. 2013. *Pellaea flavescens*, a Brazilian Endemic, is a synonym of Old World *Pellaea viridis*. **American Fern Journal 103**: 21–26.

PRADO, J. & HIRAI, R.Y. 2014. Biogeography of the Brazilian Atlantic Forest: Evidence from phylogenetic data sets and perspectives for fern and lycophyte studies. **Fern Gazette 19**: 241–257.

PRADO, J., SYLVESTRE, L.S., LABIAK, P.H., WINDISCH, P.G., SALINO, A., BARROS, I. C.L., HIRAI, R.Y., ALMEIDA, T.E., SANTIAGO, A.C.P.,

KIELING–RUBIO, M.A., PEREIRA, A.F.N., ØLLGAARD, B., RAMOS, C.G.V., MICKEL, J.T., DITTRICH, V.A.O., MYNSSEN, C.M., SCHWARTSBURD, P.B., CONDACK, J.P.S., PEREIRA, J.B.S. & MATOS, F.B. 2015. Diversity of ferns and lycophytes in Brazil. **Rodriguésia 66**: 1073–1083.

PRYER, K.M., SCHUETTPELZ, E., WOLF, P.G., SCHNEIDER, H., SMITH, A.R. & CRANFILL, R. 2004. Phylogeny and evolution of ferns (monilophytes) with a focus on the early leptosporangiate divergences. **American Journal of Botany 91**: 1582–1598.

QIAGEN, 2006. DNEasy Plant Mini Kit DNA extraction protocol. QIAGEN.

RADFORD, A.E., DICKSON, W.C., MASSEY, R. & BELL, C.R. 1974. **Vascular Plant Systematic**. Hayer & Row, New York.

RAMOS, C.G.V. & SYLVESTRE, L.S. 2010. Lycopodiaceae no Parque Nacional do Itatiaia, RJ e MG, Brasil. **Acta Botanica Brasilica 24**: 25–46.

RANKER, T.A., GEIGER, J.M.O., KENNEDY, S.C., SMITH, A.R., HAUFLER, C.H. & PARRIS, B.S. 2003. Molecular phylogenetics and evolution of the endemic Hawaiian genus *Adenophorus* (Grammitidaceae). **Molecular Phylogenetics and Evolution 26**: 337–347.

RANKER, T.A., SMITH, A.R., PARRIS, B.S., GEIGER, J.M., HAUFLER, C.H., STRAUB, S.C.K. & SCHENIDER, H. 2004. Phylogeny and evolution of grammitid ferns (Grammitidaceae): A case of rampant morphological homoplasy. **Taxon 53**: 415–428.

RYDIN, C. & WIKSTRÖM, N. 2002. Phylogeny of *Isoëtes* (Lycopsida): Resolving basal relationships using *rbcL* sequences. **Taxon 51**: 83–89.

SAFFORD, H.F. 2007. Brazilian Páramos IV. Phytogeography of the campos de

altitude. **Journal of Biogeography 34**: 1701–1722.

SCHNEIDER, H., SMITH, A.R., CRANFILL, R., HAUFLER, C.H., RANKER, T.A. & HILDEBRAND, T. 2002. *Gymnogrammitis dareiformis* is a polygrammoid fern (Polypodiaceae) – Resolving an apparent conflict between morphological and molecular data. **Plant Systematics and Evolution 234**: 121–136.

SKOG, J.E., MICKEL, J.T., MORAN, R.C., VOLOVSEK, M. & ZIMMER, E.A. 2004. Molecular studies of representative species in the fern genus *Elaphoglossum* (Dryopteridaceae) based on cpDNA sequences *rbcL*, *trnL–F*, and *rps4–trnS*. **International Journal of Plant Sciences 165**: 1063–1075.

SCHUETTPELZ, E. & PRYER, K.M. 2007. Fern phylogeny inferred from 400 leptosporangiate species and three plastid genes. **Taxon 56:** 1037–1050.

SMITH, A.R., PRYER, K.M., SCHUETTPELZ, E., KORALL, P., SCHNEIDER, H. & WOLF, P.G. 2006. A classification for extant ferns. **Taxon 55**: 705–731.

SMITH, A.R., PRYER, K.M., SCHUETTPELZ, E., KORALL, P., SCHNEIDER, H. & WOLF, P.G. 2008. Fern classification: 417–467. In: T.A. Ranker & C.H. Haufler (eds.), **Biology and evolution of ferns and lycophytes**. Cambridge University Press, Cambridge.

STAMATAKIS, A., HOOVER, P., & ROUGEMONT, J. 2008. A rapid bootstrap algorithm for the RAxML web servers. **Systematic biology 57:** 758–771.

STAMATAKIS, A. 2014. RAxML Version 8: A tool for Phylogenetic Analysis and Post–Analysis of Large Phylogenies. **Bioinformatics 10**: 1093/bioinformatics/btu033

SUNDUE, M.A., TESTO, W.L., & RANKER, T.A., 2015. Morphological innovation, ecological opportunity, and the radiation of a major vascular epiphyte lineage. **Evolution 69**: 2482–2495.

SWOFFORD, D.L. 2002. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. **Sinauer Associates**, Sunderland, Massachusetts.

TABERLET, P., GIELLY, L., PAUTOU, G., & BOUVET, J. 1991. Universal primers for amplification of 3 noncoding regions of chloroplast DNA. **Plant Molecular Biology 17**: 1105–1109.

TANG, X.C. & HAN, Y.F. 1999. Pharmacological profile of huperzine A, a novel acetylcholinesterase inhibitor from Chinese herb. **CNS Drug Reviews 5**: 281–300.

TRYON, R.M. 1972. Endemic areas and geographic speciation in Tropical American Ferns. **Biotropica 4**: 121–131.

TRYON, R.M. & TRYON, A.F. 1982. **Ferns and allied plants, with special reference to tropical America**. Springer Verlag, New York, 857 p.

VALLEJO, M., LOYOLA, S., CONTRERAS, D., UGARTE, G., CIFUENTE, D. ORTEGA, G. & DELGADO, R. 2014. A new semisynthetic derivative of sauroine induces LTP in hippocampal slices and improves learning performance in the Morris water maze. **Journal of neurochemistry 129**: 864–876.

WAGNER, W.H. JR. & BEITEL, J.M. 1992. Generic classification of modern North American Lycopodiaceae. **Annals of the Missouri Botanical Garden 79**: 676–686.

WIKSTRÖM, K. 2001. Diversification and relationships of extant homosporous Lycopods. **American Fern Journal 91**: 150–165.

WIKSTRÖM, K. & KENRICK, P. 1997. Phylogeny of Lycopodiaceae (Lycopsida) and the relationships of *Phylloglossum drummondii* Kunze based on *rbcL*

sequences. **International Journal of Plant Sciences 158**: 862–871.

WIKSTRÖM, K. & KENRICK, P. 2000a. Phylogeny of epiphytic *Huperzia* (Lycopodiaceae): Paleotropical and neotropical clades corroborated by *rbcL* sequences. **Nordic Journal of Botany 20**: 165–171.

WIKSTRÖM, K. & KENRICK, P. 2000b. Relationships of *Lycopodium* and *Lycopodiella* based on combined plastid *rbcL* gene and *trnL* intron sequence data. **Systematic Botany 25**: 495–510.

WIKSTRÖM, K. & KENRICK, P. 2001. Evolution of Lycopodiaceae (Lycopsida): Estimating divergence times from *rbcL* gene sequences by use of nonparametric rate smoothing. **Molecular Phylogenetics and Evolution 19**: 177–186.

WIKSTRÖM, N., KENRICK, P. & CHASE, M. 1999. Epiphytism and terrestrialization in tropical *Huperzia* (Lycopodiaceae). **Plant Systematics and Evolution 218**: 221–243.

WINDISCH, P.G. & ØLLGAARD, B. 2014. Lycopodiaceae in Brazil. Conspectus of the family I. The genera *Lycopodium*, *Austrolycopodium*, *Diphasium*, and *Diphasiastrum*. **Rodriguésia 65**: 293–309.

YATSENTYUK, S.P., VALIEJO–ROMAN, K.M., SAMIGULLIN, T.H., WILKSTRÖM, N. & TROITSKY, A.V. 2001. Evolution of Lycopodiaceae inferred from spacer sequencing of chloroplast rRNA Genes. **Russian Journal of Genetics 37**: 1068–1073.

ZHANG, L. & IWATSUKI, K. 2013. Lycopodiaceae: 13–34. In: A.I. Al–Shehbaz *et al.,* (org.), **Flora of China – Lycopodiaceae through Polypodiaceae**. 1[a] ed. Missouri Botanical Garden, St. Louis.

Appendix 1

List of the taxa and specimens used in this study and GenBank accession numbers for *rbc*L, *trn*L, and *trn*L-F.
Missing sequences are indicated by a dash.

Species	Marker	Marker	Marker
LOCATION Collector & number (Herbarium)	*rbc*L	*trn*L	*trn*L-F
Huperzia selago (L.) Bernh. ex Schrank & Mart.			
SWEDEN: N. Wikstrom *36* (S)	Y07934	AJ224592	AJ224592
Huperzia serrata (Thunb. ex Murray) Trevis.			
CHINA: S.L. Pan *WJ409041* (SHMU)	DQ464228	–	–
Phlegmariurus acerosus (Sw.) B. Øllg.			
BRAZIL – SÃO PAULO: J. Prado *2349* (SP, AAU)	This study	This study	This study
Phlegmariurus attenuatus (Spring) B. Øllg.			
ECUADOR: N. Wikstrom *300* (S)	–	AJ224573	AJ224573
COSTA RICA: W. Testo *167* (VT)	KT634232	–	–
Phlegmariurus biformis (Hook.) B. Øllg.			
BRAZIL – SÃO PAULO: D.S. Gissi *159* (SP, AAU)	This study	This study	This study
BRAZIL – RIO DE JANEIRO: D.S. Gissi *129* (SP, AAU)			
Phlegmariurus campianus (B. Øllg.) B. Øllg.			
ECUADOR: B. Øllgaard *100612* (AAU)	X98282	AJ224586	AJ224586
Phlegmariurus christii (Silveira) B. Øllg.			
BRAZIL – RIO DE JANEIRO: D.S. Gissi *109* (SP, AAU)	This study	This study	This study
BRAZIL – RIO DE JANEIRO: D.S. Gissi *110* (SP, AAU)			
Phlegmariurus comans (Herter ex Nessel) B. Øllg.			
BRAZIL – RIO DE JANEIRO: D.S. Gissi *100* (SP, AAU)	This study	This study	This study
BRAZIL – RIO DE JANEIRO D.S. Gissi *107* (SP, AAU)			
Phlegmariurus compactus (Hook) B. Øllg.			
ECUADOR: N. Wikstrom *271* (S)	–	AJ224571	AJ224571
Phlegmariurus crassus (Humb. & Bonpl. ex Willd.) B. Øllg.			
ECUADOR: B. Øllgaard *100832* (AAU)	–	AJ224572	AJ224572
Phlegmariurus cumingii (Nessel) B. Øllg.			
ECUADOR: B. Øllgaard *100836* (AAU)	Y07930	AJ224578	AJ224578
Phlegmariurus deminuens (Herter) B. Øllg.			
BRAZIL – MINAS GERAIS: D.S. Gissi *229* (SP, AAU)	This study	This study	This study
BRAZIL – MINAS GERAIS: D.S. Gissi *230* (SP, AAU)			
Phlegmariurus dichotomus (Jacq.) W.H. Wagner			
MEXICO: Eliasson *538* (S)	–	AJ224567	AJ224567
Phlegmariurus ericifolius (C. Presl) B. Øllg.			
ECUADOR: N. Wikstrom *286* (S)	–	AJ224587	AJ224587
Phlegmariurus erythrocaulon (Fée) B. Øllg.			
BRAZIL – SÃO PAULO: D.F. Peralta *4179* (SP)	This study	This study	This study

Phlegmariurus eversus (Poir.) B. Øllg.

ECUADOR: B. Øllgaard *100620* (AAU) | – | AJ224579 | AJ224579

Phlegmariurus flexibilis (Fée) B. Øllg.

BRAZIL – SÃO PAULO: R.Y. Hirai *175* (SP, AAU) | This study | This study | This study

Phlegmariurus fontinaloides (Spring) B. Øllg.

BRAZIL – SÃO PAULO: D.S. Gissi *153* (SP, AAU) | This study | This study | This study

Phlegmariurus funiformis (Cham. ex Spring) B. Øllg.

ECUADOR: Asplund *19290* (S) | – | AJ224560 | AJ224560

Phlegmariurus hemleri (Nessel) B. Øllg.

BRAZIL – RIO DE JANEIRO: D.S. Gissi *203* (SP, AAU) | This study | This study | This study

Phlegmariurus heterocarpon (Fée) B. Øllg.

BRAZIL – SÃO PAULO: J. Prado *2348* (SP, AAU) | This study | This study | This study

BRAZIL – RIO DE JANEIRO: D.S. Gissi *131* (SP, AAU)

Phlegmariurus heteroclitus (Desv. ex Poir.) B. Øllg.

ECUADOR: N. Wikstrom *283* (S) | – | AJ224588 | AJ224588

ECUADOR: M. Lehnert *1851* (VT) | KT634230 | – | –

Phlegmariurus hexastichus (B. Øllg. & P.G. Windisch) B. Øllg.

BRAZIL – SÃO PAULO: D.S. Gissi *148* (SP, AAU) | This study | This study | This study

Phlegmariurus hippurideus (Christ) B. Øllg.

ECUADOR: B. Øllgaard *100619* (AAU) | Y07931 | AJ224566 | AJ224566

Phlegmariurus hippuris (Desv. ex Poiret) A.R. Field & Testo

INDONESIA: N. Wikstrom *et al., 157* (S) | AJ133895 | AJ224550 | AJ224550

Phlegmariurus hystrix (Herter) B. Øllg.

ECUADOR: N. Wikstrom *294* (S) | – | AJ224574 | AJ224574

Phlegmariurus intermedius (Trevisan) B. Øllg.

BRAZIL – MINAS GERAIS: D.S. Gissi *175* (SP, AAU) | This study | This study | This study

Phlegmariurus lindenii (Spring) B. Øllg.

ECUADOR: B. Øllgaard *100831* (AAU) | – | AJ224585 | AJ224585

Phlegmariurus loefgrenianus (Silveira) B. Øllg.

BRAZIL – SÃO PAULO: J. Prado *2343* (SP, AAU) | This study | This study | This study

BRAZIL – SÃO PAULO: J. Prado *2342* (SP, AAU)

Phlegmariurus mandiocanus (Raddi) B. Øllg.

BRAZIL – RIO DE JANEIRO J. Prado *2399* (SP) | This study | This study | This study

Phlegmariurus martii (Wawra) B. Øllg.

BRAZIL – BAHIA: F.B. Matos *1401* (SP, NY, UPCB) | This study | This study | This study

Phlegmariurus mingcheensis Ching

CHINA: S.L. Pan *WJ112127* (SHMU) | DQ464232 | – | –

Phlegmariurus mollicomus (Spring) B. Øllg.

BRAZIL – RIO DE JANEIRO: C.M. Mynssen *1147* (SP, RB) | This study | This study | This study

61

Phlegmariurus nudus (Nessel) B. Øllg.

BRAZIL – RIO DE JANEIRO: D.S. Gissi *106* (SP, AAU) | This study | This study | This study

Phlegmariurus ophioglossoides (Lam.) A.R. Field & Bostock

TANZANIA: S. Manktelow *91135* (UPS) | – | AJ224590 | AJ224590

Phlegmariurus petiolatus (C. B. Clarke) H. S. Kung & Li Bing Zhang

CHINA: S.L. Pan *WJ107181* (SHMU) | DQ464233 | – | –

Phlegmariurus phlegmaria (L.) T. Sen & U. Sen

JAPAN – (TNS:763399) | AB574635 | – | –

INDONESIA: N. Wikstrom *160* (S) | – | AJ224558 | AJ224558

Phlegmariurus polydactylus (B. Øllg.) B. Øllg.

ECUADOR: B. Øllgaard *100834* (AAU) | – | AJ224575 | AJ224575

Phlegmariurus pungentifolius (Silveira) B. Øllg.

BRAZIL – MINAS GERAIS: D.S. Gissi *176* (SP, AAU) | This study | This study | This study

Phlegmariurus quadrifariatus (Bory) B. Øllg.

BRAZIL – RIO DE JANEIRO: D.S. Gissi *130* (SP, AAU) | This study | This study | This study

BRAZIL – RIO DE JANEIRO: D.S. Gissi *261* (SP)

Phlegmariurus recurvifolius (Rolleri) B. Øllg.

BRAZIL – MINAS GERAIS: D.S. Gissi *177* (SP, AAU) | This study | This study | This study

Phlegmariurus reflexus (Lam.) B. Øllg.

ECUADOR: B. Øllgaard *100617* (AAU) | | AJ224581 | AJ224581

MEXICO: M. Sundue *3089* (AAU, MEXU, VT) | KT634233

Phlegmariurus reflexus (Lam.) B. Øllg.

BRAZIL – SÃO PAULO: D.S. Gissi *165* (SP, AAU) | This study | This study | This study

BRAZIL – MINAS GERAIS: D.S. Gissi *233* (SP, AAU)

BRAZIL – SÃO PAULO: D.S. Gissi *155* (SP, AAU)

Phlegmariurus regnellii (Maxon) B. Øllg.

BRAZIL – MINAS GERAIS: E.A. Williams *469* (RB) | This study | This study | This study

Phlegmariurus rosenstockianus (Herter) B. Øllg.

ECUADOR: J. Branbyge *42178* (S) | – | AJ224583 | AJ224583

Phlegmariurus rufescens (Hook.) B. Øllg.

ECUADOR: B. Øllgaard *100833* (AAU) | – | AJ224576 | AJ224576

Phlegmariurus sarmentosus (Spring) B. Øllg.

ECUADOR: B. Øllgaard *100816* (AAU) | – | AJ224584 | AJ224584

Phlegmariurus silveirae (Nessel) B. Øllg.

BRAZIL – MINAS GERAIS: V.A.O. Dittrich *1812* (CESJ) | This study | This study | This study

Phlegmariurus squarrosus (G. Forst.) Á. Löve & D. Löve

CHINA: S.L. Pan WJ207111 (SHMU) | DQ464235 | – | –

MALAYSIA: N. Wikstrom *143* (S) | – | AJ224557 | AJ224557

Phlegmariurus subulatus (Desv. ex Poir.) B. Øllg.

ECUADOR: B. Øllgaard *100618* (AAU)	–	AJ224589	AJ224589
Phlegmariurus tenuis (Humb. & Bonpl. Ex Willd.) B. Øllg.			
ECUADOR: B. Øllgaard *100817* (AAU)	–	AJ224568	AJ224568
Phlegmariurus tetragonus (Hook. & Grev.) B. Øllg.			
ECUADOR: N. Wikstrom *301* (S)	–	AJ224577	AJ224577
Phlegmariurus unguiculatus (B. Øllg.) B. Øllg.			
ECUADOR: N. Wikstrom *264* (S)	–	AJ224582	AJ224582
Phlegmariurus verticillatus (L.f.) A.R. Field & Testo			
INDONESIA: N. Wikstrom *et al., 156* (S)	AJ133897	AJ224561	AJ224561
Phlegmariurus wilsonii (Underw. & F.E. Lloyd) B. Øllg.			
ECUADOR: B. Øllgaard *et al., 100611* (AAU)	Y07933	AJ224565	AJ224565
Phylloglossum drummondii Kunze			
AUSTRALIA: Crane *s.n.* (S)	Y07939	AJ224593	AJ224593