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Molecular phylogeny of bladderworts: A wide approach of *Utricularia* (Lentibulariaceae) species relationships based on six plastidial and nuclear DNA sequences

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ABSTRACT

The carnivorous plant genus *Utricularia* L. (bladderwort) comprises about 240 species distributed worldwide and is traditionally classified into two subgenera (*Polypompholyx* and *Utricularia*) and 35 sections, based mainly on general and trap morphology. It is one out of the largest carnivorous genera, representing ca. 30% of all carnivorous plant species, and is also the most widely distributed. According to previous phylogenetic studies, most infrageneric sections are monophyletic, but there are several incongruences considering their relationships and also the dissenting position of some species as a result of a too few (mostly one or two) molecular markers analyzed. Thus, here we present a multilocus phylogeny for *Utricularia* species with a wide taxonomic sampling (78 species and 115 accessions) based on six plastid (*rbcL*, *matK*, *rpl20-rps12*, *rps16*, *trnL-F*) and nuclear DNA (ITS region) sequences. The aim is to reconstruct a well-resolved tree to propose evolutionary and biogeographic hypotheses for the radiation of lineages with inferences about the divergence times of clades using a molecular clock approach.

1. Introduction

The family Lentibulariaceae comprises three genera: *Utricularia* L., *Genlisea* A.St.-Hil. and *Pinguicula* L.; all of which are carnivorous herbs. The genus *Utricularia* comprises plants known as bladderworts and holds about 240 species traditionally classified in two subgenera and 35 sections since Taylor's monograph (1989; Fleischmann, 2015; Rutishauser, 2016). It is one out of the largest carnivorous plant genera and is also the most widely distributed, representing ca. 30% of all carnivorous plants taxa (Król et al., 2012). Bladderworts are one of the most intriguing carnivorous plants, with traps of such structural and physiological complexity that they are still not fully understood (Juniper et al., 1989; Rutishauser and Isler, 2001; Poppinga et al., 2015). The traps, known as utricles, are leaf-like structures shaped in small vesicles, which are active in prey capture and secretion of hydrolytic enzymes for digesting small animals (Adamec et al., 2010; Król et al., 2012; Lloyd, 1935; Poppinga et al., 2015; Sirová et al., 2003, 2009).

All *Utricularia* species are herbs and their vegetative structures do not follow traditional models of morphological classification. The archetypes of body organization of *Utricularia* do not present clearly defined boundaries between typical plant organs (e.g. stems, leaves) and these plants do not have roots (Rutishauser and Isler, 2001). Bladderworts are adapted to colonize different environments, from land with terrestrial, lithophyte and epiphyte species to open water with free-floating and affixed aquatic species, rheophytes, and epiphytes (Taylor, 1989).

The taxonomic system proposed by Taylor (1989), still the most important taxonomic reference to the genus, is based mainly on morphological characters; especially reproductive structures (calyx, corolla, peduncle and seed) and traps. *Utricularia* shows a great phenotypic plasticity, both intra and inter-specific, and hence species are often taxonomically difficult to identify.

Bladderworts have attracted special interest by many researchers

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since the recent discovery that both *Utricularia* and *Genlisea* have species with the smallest genomes currently known within the angiosperms (Ibarra-Laclette et al., 2013). The chromosomes sizes of some species are comparable to the size of bacteria (Fleischmann et al., 2014; Greilhuber et al., 2006) and there is a wide variation in genome size within genera and species (Veleba et al., 2014). Also, high rates of mutation among all intracellular compartments have also been reported (Jobson and Albert, 2002; Müller and Borsch, 2005). Therefore, *Utricularia* and *Genlisea* species can be extraordinarily suitable model plants for understanding the process of miniaturization of genomes (Albert et al., 2010; Fleischmann et al. 2014; Greilhuber et al. 2006; Ibarra-Laclette et al., 2011, 2013). This primarily applies to the range of genome sizes found within both genera. It is remarkable that ultra-small genomes (1C < 100 Mbp) have evolved independently in at least three independent lineages in the genus *Utricularia* (Veleba et al., 2014).

According to previous phylogenetic studies, in general, most sections proposed by Taylor (1989) are monophyletic. Nevertheless, there are several incongruences between the clades, considering their relationships and also the dissenting position of some species. In attempt to solve these issues, Müller et al. (2006) used the supertrees approach, however important questions still remained unsolved, such as the phylogenetic interrelationship of *Utricularia* subgenera *Polypompholyx* and *Utricularia,* which is still ambiguous. Thus the need of a broad phylogenetic analysis is evident, mainly of *Utricularia* species. The previously published studies focused only on a few markers, and then they were primarily partial sequences from cpDNA such as *matK*, *rps16*, *trnL-F* and *trnK* (Jobson and Albert, 2002; Jobson et al., 2003; Müller and Borsch, 2005; Reut and Jobson, 2010; Westermeier et al., 2017). Therefore, a robust phylogenetic analysis of *Utricularia* species is clearly needed, with a premise that a wide taxonomic sampling with several DNA markers could untangle the evolutionary relationships of bladderworts.

Here we evaluate a multilocus phylogeny for *Utricularia* species with no precedents, including both the wide taxonomical representation (78 species and 115 accessions of *Utricularia*) and also the DNA markers sampled (five from plastid and one region from the nuclear genome). We aimed to: (1) reconstruct a well-resolved tree based on five chloroplast sequences from the plastid genome (*rbcL*, *matK*, *rpl20-rps12, rps16, trnL-trnF*) and one from the nuclear genome (ITS rDNA: ITS1 + 5.8S + ITS2); (2) propose an evolutionary hypothesis for the radiation of lineages and species in different habitats; (3) propose a biogeographical hypothesis for the *Utricularia* lineages; and, (4) infer the divergence time of clades using a molecular clock approach.

2. Materials and methods

2.1. Plant material

This study comprises 115 accessions representing 78 *Utricularia* species, resulting from leaves and flowers stored in silica gel samples collected from natural populations (mainly from Brazil and Australia), from cultivated specimens in Unesp/FCAV Jaboticabal, Brazil, and in the Aquatic and Wetland Plant Collection from the Institute of Botany, Třeboň, Czech Republic, and available sequences from GenBank (www.ncbi.nlm.gov; Table 1). The sectional classification of the genus *Utricularia* was followed as proposed by Taylor (1989). *Genlisea* and *Pinguicula* species were selected as the outgroup. Vouchers were deposited for all materials collected for this study in Herbarium JABU (Unesp/FCAV campus Jaboticabal, Brazil) and the National Herbarium of New South Wales (NSW).

2.2. Life forms and geographic distribution of taxa

The data about the life forms of all taxa was adopted from Taylor (1989) and also our own observations of the species in nature. Therefore, to simplify the analysis and trace possible ancestral forms, we have adapted the life form categories from Taylor's classification (1989), making no distinction between the different aquatic habits (e.g., free-floating and affixed aquatic species were considered as aquatic) and also considering lithophyte as terrestrial species. Thus, we considered only four life forms: aquatic, epiphyte, rheophyte and terrestrial. The geographic distribution of each species was also based on Taylor (1989).

2.3. DNA extraction and polymerase chain reaction (PCR)

We performed the DNA extractions according to the Doyle and Doyle (1987) protocol with modifications by Lodhi et al. (1994) for fresh plant tissue and dehydrated samples from silica gel preserved specimens. The amplified chloroplast regions were *rbcL* (using primers *rbcLa*–F, *rbcLa*–R; Kress et al., 2009; Levin et al., 2003), *matK* (3F– KIM and 1RKIM; Li et al., 2011), the intergenic spacer *rpl*20-*rps12* (5′-*rps*12 and *rpl*20; Hamilton, 1999) and the ITS region (ITS4 and ITS5; White et al., 1990) from the rDNA.

PCRs were achieved using Taq DNA polymerase (Thermo Scientific Fermentas®, Pittsburgh, PA, USA) containing 20 mM $MgCl_2$, nucleotides, buffer, including reactions with the adjuvant dimethyl sulfoxide (DMSO) when necessary, as recommended by Miranda et al. (2010) (for PCR and primers specifications see Table 2).

2.4. DNA sequencing and alignment

DNA sequencing was performed using Applied Biosystems ABI 3100. All chromatograms obtained from the sequences were analyzed for possible sequencing errors using the program BioEdit version 7.0.9.0 (Hall, 1999). Both forward and reverse frames were used for each sample for the construction of a consensus sequence. Several species were sequenced with bioreplicates; mostly specimens from distinct populations (see Table 1).

The sequence alignments were performed using MAFFT version 7 (Katoh and Standley, 2013) with the application of default parameters (FFT-NS-1, FFT-NS-2, FFT-NS-i or L-INS-i) and GAPs with default penalties. For the ITS matrix, due to sequences with low overlap to other alignments (with some distance pairwise values below 40%), the software MaxAlign Server 1.1 (Gouveia-Oliveira et al., 2007) was employed to identify and remove sequences that caused too much disruption in the alignment by excess of saturation. Indels were treated as missing data.

2.5. Phylogenetic analyses – maximum parsimony, maximum likelihood, and Bayesian approach

Previous phylogenetic work on the Lentibulariaceae placed the clade *Utricularia–Genlisea* with *Pinguicula* as the sister group (Jobson and Albert, 2002; Müller et al., 2004). Therefore, species of *Genlisea* and *Pinguicula* were used as outgroup (Table 1) for this study. Maximum parsimony analyses (MP) were performed with PAUP∗ version 4b10 software (Swofford, 2002). The most parsimonious trees were found by heuristic searches with random addition of sequences and 2000 replicates using the branch swapping algorithm Nearest Neighbor Interchange (NNI; Robinson, 1971). Clade support was assessed using bootstrap analysis (Felsenstein, 1985) with 2000 pseudoreplicates and heuristic search with 1000 replicates (with random addition of sequences with the branch swapping algorithm NNI). For probabilistic analyses, the best evolutionary models (best-of-fit) were searched for using jModelTest software version 2.1.1 (Santorum et al., 2014). Thus, the best-of-fit DNA model was evaluated for each data matrix with the corrected Akaike information criterion (AICc, Akaike, 1973; Burnham and Anderson, 2002) to estimate the parameters. The algorithm Metropolis-coupled Markov chain Monte Carlo (MCMCMC; Geyer, 1991); maximum likelihood (ML), and Bayesian analyses were performed to estimate the phylogenetic hypothesis for each data matrix. The ML

Table 1
Utricularia species included in this study and GenBank accession numbers by molecular marker. The *Utricularia* sections are according to Taylor (1989). Vouchers are deposited in Herbarium JABU and NSW (denoted between parentheses). (Abbreviations for collectors' names: CGM = C.G.Menezes; RG = R.Gibson; SRS = S.R.Silva; VFOM = V.F.O.Miranda; and YDR = Y.D.Rodríguez).

Species	Section	*rbcL*	*matK*	*rpl20-rps12*	*rps16*	*trnL-F*	ITS	Voucher
Utricularia multifida R.Br.	*Polypompholyx* (Lehm.) P.Taylor	MF991571	AF531848.1, KX604226	MG027801	AF482583.1	AF482659.1	–	RG500 (NSW)
Utricularia blanchetii A.DC.	*Aranella* (Barnhart) P.Taylor	–	AF531841.1	–	–	–	–	
Utricularia costata P.Taylor	*Aranella* (Barnhart) P.Taylor	AY128628.1	–	–	AF482565.1	AF482639.1	–	
Utricularia laciniata A.St.-Hil. & Girard	*Aranella* (Barnhart) P.Taylor	MF991511, MF991512,MF991513	KX604228	MG027802	AF482577.1	AF482653.1	–	VFOM1624 (JABU); VFOM1632 (JABU)
Utricularia parthenopipes P.Taylor	*Aranella* (Barnhart) P.Taylor	–	AF531842.1	–	–	–	–	
Utricularia purpureocaerulea A.St.-Hil. & Girard	*Aranella* (Barnhart) P.Taylor	–	–	–	AF482594.1	AF482670.1	–	
Utricularia simulans Pilg.	*Aranella* (Barnhart) P.Taylor	MF991543	–		AF482597.1	AF482674.1	–	VFOM1564 (JABU); VFOM1612 (JABU)
Utricularia delicatula Cheeseman	*Australes* P.Taylor	–	–	–	–	AF488530.1	–	
Utricularia lateriflora R.Br.	*Australes* P.Taylor	MF991514	KX604175	MG027799	AF482578.1	AF482654.1	MG027736	RG545 (NSW)
Utricularia simplex R.Br.	*Australes* P.Taylor					AF482673.1		
Utricularia neottioides A.St.-Hil. & Girard	*Avesicaria* Kamiénski	MF991522, MF991523	KX604193, KX604194, KX604195	MG027833, MG027834, MG027835, MG027836	AF482587.1	AF482663.1	MG027737	VFOM1307 (JABU); VFOM1629 (JABU); VFOM1640 (JABU)
Utricularia oliveriana Steyerm.	*Avesicaria* Kamiénski	–	–	–	AF482590.1	AF482666.1	–	
Utricularia rigida Benj.	*Avesicarioides* Komiya	–	AF531838.1				–	
Utricularia nana A.St.-Hil. & Girard	*Benjaminia* P.Taylor	MF991520	AF531837.1	MG027857	AF482585.1	AF482661.1	–	VFOM1593 (JABU); VFOM1599 (JABU)
Utricularia arenaria A.DC.	*Calpidisca* (Barnhart) Komiya	MF991476,MF991477,MF991478,MF991479,MF991480	KX604173	MG027794, MG027795, MG027796	–	–	MG027729, MG027730, MG027731, MG027732, MG027733	SRS11 (JABU); SRS12 (JABU); SRS13 (JABU); SRS14 (JABU); SRS15 (JABU); SRS16 (JABU)
Utricularia bisquamata Schrank	*Calpidisca* (Barnhart) Komiya	–	–	–	AF482562.1	AF482635.1	–	
Utricularia livida E.Mey.	*Calpidisca* (Barnhart) Komiya	MF991569	AF531833.1	MG027797	AF482579.1	AF482655.1	–	RG477 (NSW)
Utricularia sandersonii Oliv.	*Calpidisca* (Barnhart) Komiya	MF991541, MF991542	AF531847.1, KX604174	MG027798	AF482596.1	AF482672.1	MG027734, MG027735	SRS17; RG467 (NSW)

(*continued on next page*)

Table 1 (*continued*)

Species	Section	*rbcL*	*matK*	*rpl20-rps12*	*rps16*	*trnL-F*	ITS	Voucher
Utricularia chrysantha R.Br.	*Enskide* (Raf.) P.Taylor	–	–	–	–	AF482637.1	–	
Utricularia amethystina Salzm. ex A.St.-Hil. Girard	*Foliosa* Kamiénski	MF991574,MF991575,MF991576,MF991577,MF991578,MF991579	KX604213, KX604208	MG027791, MG027792, MG027793	AF482557.1, MG027707	AF482630.1	MG027717, MG027718, MG027719, MG027720, MG027721, MG027722, MG027723	VFOM1614 (JABU); VFOM1615 (JABU);VFOM1622 (JABU); VFOM1627 (JABU)VFOM1647 (JABU)
Utricularia tricolor A.St.-Hil.	*Foliosa* Kamiénski	MF991554,MF991555,MF991556,MF991557,	KX604209, KX604211, KX604210	–	AF482600.1	AF482677.1	MG027724, MG027725, MG027726, MG027727, MG027728	VFOM1536 (JABU); VFOM1540 (JABU); VFOM1546 (JABU); SRS34 (JABU)
Utricularia tridentata Sylvén	*Foliosa* Kamiénski	MF991566	AF531825.1, KX604212	–	–	–	–	VFOM S/N (JABU)
Utricularia geminiloba Benj.	*Iperua* P.Taylor	–	KX604216			AF482646.1	MG027775	CGM42 (JABU)
Utricularia humboldtii R.H.Schomb.	*Iperua* P.Taylor	–	AF531836.1	–	–	–	–	
Utricularia nelumbifolia Gardner	*Iperua* P.Taylor	MF991521	KX604217	MG027850	AF482586.1	AF482662.1	–	SRS18 (JABU)
Utricularia nephrophylla Benj.	*Iperua* P.Taylor	–	AF531827.1		AF482588.1	AF482664.1	–	
Utricularia reniformis A.St.-Hil.	*Iperua* P.Taylor	MF991536, MF991537, MF991538, MF991539	AF531828.1, KX604220, KX604219,NNNNNN	MG027851, MG027852, MG027853,MG027854	AF482595.1	AF482671.1	MG027776, MG027777, MG027778, MG027779	VFOM1500 (JABU); VFOM1559 (JABU)
Utricularia resupinata B.D.Greene ex Bigelow	*Lecticula* (Barnhart) Komiya	MF991540	–	MG027828	AF488527.1	AF488533.1	MG027767	CP25826 (JABU)
Utricularia pubescens Sm.	*Lloydia* P.Taylor	AY128629.1	AF531844.1	–	AF482593.1	AF482669.1	–	
Utricularia biloba R.Br.	*Nelipus* (Raf.) P.Taylor	–	FN773562.1	–	AF482561.1	AF482634.1	–	
Utricularia leptoplectra F.Muell.	*Nelipus* (Raf.) P.Taylor	–	–	–	AF488526.1	AF488532.1	–	
Utricularia caerulea L.	*Nigrescentes* (Oliver) Komiya	–	–	–	–	AF482636.1	–	
Utricularia adpressa Salzm. ex A.St.-Hil. & Girard	*Oligocista* A.DC.	–	–	–	–	AF482628.1	–	
Utricularia bifida L.	*Oligocista* A.DC.	MF991483	KX604229	MG027855			MG027739	VFOM S/N (JABU)
Utricularia erectiflora A.St.-Hil. & Girard	*Oligocista* A.DC.	–	–	–	AF482568.1	AF482643.1	–	
Utricularia foveolata Edgew.	*Oligocista* A.DC.	–	AF531850.1	–	AF482570.1	AF482645.1	–	

(*continued on next page*)

Table 1 (*continued*)

Species	Section	*rbcL*	*matK*	*rpl20-rps12*	*rps16*	*trnL-F*	ITS	Voucher
Utricularia graminifolia Vahl	*Oligocista* A.DC.	–	–	–	AF482573.1	AF482649.1	–	
Utricularia laxa A.St.-Hil. & Girard	*Oligocista* A.DC.	MF991568	–	–	–	–	–	VFOM1537 (JABU); VFOM1555 (JABU)
Utricularia meyeri Pilg.	*Oligocista* A.DC.	MF991570	KX604231	MG027856	AF482582.1	AF482658.1	–	CGM81 (JABU)
Utricularia prehensilis E.Meyer	*Oligocista* A.DC.	–	–	–	AF482592.1	AF482668.1	–	
Utricularia spiralis Sm.	*Oligocista* A.DC.	–	AF531851.1				–	
Utricularia uliginosa Vahl	*Oligocista* A.DC.	MF991562	AF531849.1	MG027858	AF482602.1	AF482679.1	–	RG568 (NSW)
Utricularia adpressa Salzm. ex A.Sil.-Hil. & Girard	*Orchidioides* A.DC.	AF482527.1	–	–	AF482555.1		–	
Utricularia alpina Jacq.	*Orchidioides* A.DC.	AF482528.1	AF531822.1	–	AF482556.1	AF482629.1	–	
Utricularia asplundii P.Taylor	*Orchidioides* A.DC.	–	–	–	AF482558.1	AF482631.1	–	
Utricularia caerulea L.	*Orchidioides* A.DC.	–	–	–	AF482563.1		–	
Utricularia endressii H.G.Reichb.	*Orchidioides* A.DC.	–	–	–	–	AF482642.1	–	
Utricularia quelchii N.E. Br.	*Orchidioides* A.DC.	–	AF531846.1	–	–	–	–	
Utricularia striatula Sm.	*Phyllaria* (Kurz) Kamieński	–	–	–	AF482598.1	AF482675.1	–	
Utricularia dichotoma Labill.	*Pleiochasia* Kamiénski	AY128632, MF991494	AF531826.1	MG027800	AF482567.2	AF482641.1	MG027737	RG220 (NSW)
Utricularia menziesii R.Br.	*Pleiochasia* Kamiénski	MF991573	–	–	–	–	–	RG309 (NSW)
Utricularia monanthos J.D.Hook.	*Pleiochasia* Kamiénski	MF991519	–	–	GQ478686.1	–	–	RG531 (NSW)
Utricularia uniflora R.Br.	*Pleiochasia* Kamiénski	MF991563	–	–	GQ478698.1	–	–	RG385 (NSW)
Utricularia violacea R.Br	*Pleiochasia* Kamiénski	–	–	–	AF482603.1	AF482680.1	–	
Utricularia calycifida Benj.	*Psyllosperma* P.Taylor	MF991489, MF991490	AF531824.1	–	–	–	MG027740, MG027741	SRS20 (JABU); VFOM1653 (JABU)
Utricularia hispida Lam.	*Psyllosperma* P.Taylor	MF991503,MF991504,MF991505,MF991506,MF991507	AF531829.1, KX604224, KX604223, KX604222, KX604221	–	–	–	MG027780, MG027781, MG027782, MG027783	VFOM1637 (JABU)
Utricularia huntii P.Taylor	*Psyllosperma* P.Taylor	–	–	–	AF482574.1	AF482650.1		
Utricularia longifolia Gardner	*Psyllosperma* P.Taylor	MF991515, MF991516	AF531834.1, KX604214, KX604215	–	AF482580.1	AF482656.1	MG027773, MG027774	CGM40 (JABU); CGM50 (JABU)
Utricularia praelonga A.St.-Hil. & Girard	*Psyllosperma* P.Taylor	MF991572, MF991529	AF531843.1, KX604225	–	AF482591.1	AF482667.1	–	VFOM1538 (JABU)

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Table 1 (*continued*)

Species	Section	*rbcL*	*matK*	*rpl20-rps12*	*rps16*	*trnL-F*	ITS	Voucher
Utricularia flaccida A.DC.	*Setiscapella* (Barnhart) P.Taylor	–	AF531830.1	–	AF482569.1	AF482644.1	–	VFOM1261 (JABU); VFOM1266 (JABU)
Utricularia nervosa Weber ex Benj.	*Setiscapella* (Barnhart) P.Taylor	MF991524, MF991525, MF991526, MF991527	KX604199, KX604198, KX604197, KX604196	MG027837, MG027838, MG027839, MG027840, MG027841	–	–	MG027760, MG027761, MG027762	VFOM1544 (JABU); VFOM1649 (JABU)
Utricularia pusilla Vahl	*Setiscapella* (Barnhart) P.Taylor	MF991532	–	–	–	–	–	VFOM1566 (JABU)
Utricularia subulata L.	*Setiscapella* (Barnhart) P.Taylor	MF991548, MF991549, MF991550, MF991551, MF991552, MF991553	AF531821.1, KX604201, KX604200, KX604203, KX604202	MG027842, MG027843, MG027844, MG027845	AF482599.1	AF482676.1	MG027763, MG027764	VFOM1616 (JABU); VFOM1625 (JABU); VFOM1630 (JABU); RG476 (NSW)
Utricularia triloba Benj.	*Setiscapella* (Barnhart) P.Taylor	AF482530.1, MF991558, MF991559, MF991560, MF991561	KX604204, KX604205, KX604206, KX604207	MG027846, MG027847, MG027848, MG027849	AF482601.1	AF482678.1	MG027765, MG027766	VFOM1587 (JABU); VFOM1631 (JABU); VFOM1634 (JABU)
Utricularia cornuta Michx.	*Stomoisia* (Raf.) Kuntze	–	–	–	AF482564.1	AF482638.1	–	
Utricularia juncea Vahl	*Stomoisia* (Raf.) Kuntze	AY128630.1	AF531832.1	–	AF482576.1	AF482652.1	–	
Utricularia volubilis R.Br.	*Tridentaria* P.Taylor	MF991564	KX604227	–	–	–	MG027738	CP25809 (JABU)
Utricularia aurea Lour.	*Utricularia* P.Taylor	MF991481	KX604176	MG027807	AF482559.1	AF482632.1	MG027742	RG440 (NSW); CP25840 (JABU)
Utricularia australis R.Br.	*Utricularia* P.Taylor	JN893443.1, JN893264.1, MF991482	AF531823.1	MG027808	AF482560.1	AF482633.1	–	CP25896 (JABU)
Utricularia bremii Heer	*Utricularia* P.Taylor	MF991484	KX604177	MG027809	–	–	MG027744	CP25792 (JABU)
Utricularia breviscapa Wright ex Griseb.	*Utricularia* P.Taylor	MF991485, MF991486, MF991487, MF991488	KX604191, KX604178	MG027821, MG027822, MG027823	–	–	MG027755, MG027756, MG027757	VFOM1476 (JABU); SRS401 (JABU); SRS402 (JABU); CP25800 (JABU)
Utricularia dimorphantha Makino	*Utricularia* P.Taylor	MF991495	KX604186	MG027812	–	–	MG027749	CP25788 (JABU)
Utricularia floridana Nash	*Utricularia* P.Taylor	MF991496	KX604192	MG027804	–	–		CP25794 (JABU)
Utricularia foliosa L.	*Utricularia* P.Taylor	MF991497, MF991498, MF991499, MF991500	KX604188, KX604187	MG027825, MG027826	MG027706		MG027750, MG027751, MG027752, MG027753, MG027754	SRS4 (JABU)
Utricularia geminiscapa Benj.	*Utricularia* P.Taylor	–	–	MG027813	AF482571.1	AF482647.1	EF526399.1	CP25856 (JABU)
Utricularia gibba L.	*Utricularia* P.Taylor	L13190.2, MF991502, MF991544	NC_021449.1	NC_021449.1, MG027803, MG027805	AF482572.1	AF482648.1	–	SRS5 (JABU)
Utricularia hydrocarpa Vahl	*Utricularia* P.Taylor	MF991508	KX604190	MG027827	–	–	MG027758	CP25799 (JABU)
Utricularia inflata Walter	*Utricularia* P.Taylor	–	–		AF488525.1	AF488531.1	–	
Utricularia inflexa Forssk.	*Utricularia* P.Taylor	MF991509		MG027819			MG027743	CP25863 (JABU)

(*continued on next page*)

Table 1 (*continued*)

Species	Section	*rbcL*	*matK*	*rpl20-rps12*	*rps16*	*trnL-F*	ITS	Voucher
Utricularia intermedia Hayne	*Utricularia* P.Taylor	JN966055.1, JN890779.1, MF991510	AF531839.1, JN894029.1, JN966728.1, KX604179	–	AF482575.1	AF482651.1	–	CP25786 (JABU)
Utricularia macrorhiza J. Le Conte	*Utricularia* P.Taylor	MF991501, MF991517	AF531835.1, KX604183	MG027814	AF482581.1	AF482657.1	MG027747	CP25832 (JABU)
Utricularia minor L.	*Utricularia* P.Taylor	KC484305.1, KC584902.1, JN891120.1, JN890553.1, MF991518	JN894028.1, JN894431.1, KC584950.1, KX604181	MG027811	–	GU169706.1	MG027745	CP25785 (JABU)
Utricularia ochroleuca R.W.Hartm.	*Utricularia* P.Taylor	MF991528	–	MG027818	–	–	MG027746	CP25891 (JABU)
Utricularia olivacea C.Wright ex Griseb.	*Utricularia* P.Taylor	MF991567	AF531840.1		AF482589.1	AF482665.1	–	CGM89 (JABU)
Utricularia radiata Small	*Utricularia* P.Taylor	MF991533	KX604185	MG027824	–	–	–	CP25838 (JABU)
Utricularia reflexa Oliv.	*Utricularia* P.Taylor	MF991534, MF991535	KX604189	MG027817, MG027820	–	–	–	CP25831 (JABU); CP25853 (JABU)
Utricularia stellaris L.f.	*Utricularia* P.Taylor	MF991545	KX604182	MG027815	–	–	–	CP25805 (JABU)
Utricularia striata Le Conte ex Torr.	*Utricularia* P.Taylor	MF991546	–	MG027806	–	–	–	CP25795 (JABU)
Utricularia stygia Thor	*Utricularia* P.Taylor	JN890776.1, MF991547	JN894027.1, KX604180	MG027816	–	–	–	CP25790 (JABU)
Utricularia vulgaris L.	*Utricularia* P.Taylor	JN890555.1, MF991565, JN890809.1	AF531831.1, JN896195.1, JN894054.1, JN966728.1	MG027810	–	JQ728994.1	EF526385.1, MG027748	CP25783 (JABU)
Utricularia myriocista A.St.-Hil. & Girard	*Vesiculina* (Raf.) P.Taylor	–	–	–	AF482584.1	AF482660.1	–	
Utricularia cucullata A.St.-Hil. & Girard	*Vesiculina* (Raf.) P.Taylor	MF991491, MF991492, MF991493		MG027829, MG027832	AF482566.1	AF482640.1	MG027768, MG027769, MG027770, MG027771, MG027772	VFOM1651 (JABU)
Utricularia purpurea Walter	*Vesiculina* (Raf.) P.Taylor	MF991530, MF991531	AF531845.1	MG027830, MG027831	–	–	–	CP25792 (JABU); CP25793 (JABU)
Genlisea aurea A.St.-Hil.		AUSU01000124.1	AF531814.1, FN641694.1, FN641695.1, FN641714.1	–	FN641746.1	AF482614.1	–	
Genlisea filiformis A.St.-Hil.		MF991463, MF991464, MF991465, MF991466	FN641690.1, FN641691.1	MG027787	FN641749.1	–	–	VFOM1633 (JABU); VFOM1641 (JABU)
Genlisea guianensis N.E.Br.		AY128631.1	–	–	AF482541.1	AF482615.1	–	
Genlisea lobata Fromm		–	FN641711.1	–	–	–	–	
Genlisea repens Benj.		–	FN641689.1	–	–	–	–	

(*continued on next page*)

Table 1 (*continued*)

Species	Section	*rbcL*	*matK*	*rpl20-rps12*	*rps16*	*trnL-F*	ITS	Voucher
Genlisea violacea A.St.-Hil.		MF991467, MF991468, MF991469, MF991470	FN641716.1, FN641717.1	MG027788, MG027789, MG027790	AF482542.1	AF482616.1	MG027712, MG027713, MG027714, MG027715, MG027716	VFOM1622 (JABU); VFOM1623 (JABU); VFOM1626 (JABU); VFOM1632 (JABU); VFOM1636 (JABU);
Pinguicula agnata Casper		AY128627.1	AF531782.1	–	AF482543.1	AF482617.1		
Pinguicula antarctica Vahl		–	DQ010653.1	–	–	–	–	
Pinguicula caerulea Walter		L01942.2	–	–	AF482545.1	AF482619.1	–	
Pinguicula ehlersiae Speta & F.Fuchs		AF482523.1	–	–	AF482547.1	AF482621.1	–	
Pinguicula filifolia C. Wright ex Griseb.		MF991471, MF991472, MF991473, MF991474, MF991475	AF531786.1, KX604168, KX604169, KX604170, KX604171, KX604172	MG027784, MG027785, MG027786	–	–	MG027708, MG027709, MG027710, MG027711	YDR31 (JABU); YDR32 (JABU); YDR41 (JABU); YDR42 (JABU); YDR S/N (JABU)
Pinguicula gracilis Zamudio		AF482524.1	–	–	AF482548.1	AF482622.1	–	
Pinguicula grandiflora Lam.		AF482525.1	–	–	AF482549.1	AF482623.1	–	
Pinguicula gypsicola Brandegee		AF482526.1	–	–	AF482550.1	AF482624.1	–	
Pinguicula lusitanica L.		JN893189.1	DQ010661.1	–	AF482551.1	AF482625.1	–	
Pinguicula moranensis Kunth		HQ384871.1	–		AF482552.1	AF482626.1	–	
Pinguicula villosa L.		JN965726.1, JN965727.1, KC483442.1	–	–	–	GQ245257.1	–	
Pinguicula vulgaris L.		KC483448.1, KC483449.1	–	–	–	GQ245258.1	–	

Table 2
Primers and PCR conditions of target sequences.

Sequence	Primer name	Primer sequence (5′–3′)	AT[a] (°C)	Extension time (s)	Reference to primers and amplification conditions
rbcL	rbcLa_f	ATGTCACCACAAACAGAGACTAAAGC	50	60	Levin (2013); this study
	rbcLa_rev	GTAAAATCAAGTCCACCRCG	50	60	Kress et al. (2009); this study
matK	3F_KIM	CGTACAGTACTTTTGTGTTTACGAG	52	20	Li et al. (2011); this study
	1R_KIM	ACCCAGTCCATCTGGAAATCTTGGTTC	52	20	Li et al. (2011); this study
rpl20-rps12	rpl20	TTTGTTCTACGTCTCCGAGC	50	60	Hamilton (1999); this study
	5′-rps12	GTCGAGGAACATGTACTAGG	50	60	Hamilton (1999); this study
ITS	ITS4	TCCTTCCGCTTATTGATATGC	50	50	White et al. (1990), Miranda et al. (2010); this study
	ITS5	GGAAGTAAAAGTCGTAACAAGG	50	50	White et al. (1990), Miranda et al. (2010); this study

[a] Annealing temperature.

analyses were run using RAxML BlackBox program (Stamatakis et al., 2008). MCMCMC analyses were performed using MrBayes software version 3.2.2 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012) for each data set with 9×10^6 generations sampled for each 100 generations, using the default parameters of the application. For each analysis two runs (nruns = 2) with four chains (nchains = 4) were performed beginning with random trees. Initial samples were discarded after reaching stationarity (estimated at 25% of the trees). The posterior probabilities (PP) for each clade were obtained by comparing and matching individual analyses with the analysis of a majority (> 50%) consensus tree. The analyses with RAxML and MrBayes applications were performed on the CIPRES resource (Miller et al., 2010). Cladograms (except the one with optimizations of ancestral states) were drawn with TreeGraph2 beta version 2.0.52–347 (Stöver and Müller, 2010) software. Further, cladograms included geographical data, the life forms of the species, and the inference of ancestral states using the software Mesquite version 2.75 (Maddison and Maddison, 2010).

2.6. Molecular clock approach

For molecular clock estimations, we used the *rbcL* dataset because out of all the sequences in this study, only *rbcL* was available for *Pinguicula filifolia* and this species was used as clock calibration point. We used the BEAST package v.1.8 (Drummond et al., 2012), with Markov chain Monte Carlo (MCMC) run for 10^6 generations, sampling for each 100 generations. The first 10% of trees were discarded as burn-in, and the remaining trees were combined in a maximum clade credibility tree using TreeAnnotator v. 1.6 (BEAST package). We used two calibration points: (i) the first was pollen grain fossils dated for *Utricularia minor* (11.6–23.0 mya; Muller, 1984) and (ii) the second was based on the insular separation of the western part of Cuba (Pinar del Río province) and Isla de la Juventud (1.8–5.3 mya; Ferrera et al., 1990) as their geological histories are very well studied. The molecular model HKY + Γ was applied for the analysis, as it was found to be the best-of-fit model using jModelTest software version 2.1.1 (Santorum et al., 2014). The priors for the analyses (other than default) were: relaxed uncorrelated lognormal clock (estimated); Yule process of speciation; and a random starting tree. To this analysis, we included a *rbcL* partial paleosequence (137 bp) found in the rumen of a Holocene Yakutian bison (*Bison priscus*) that was radiocarbon dated to about 10,500 cal a BP (Van Geel et al., 2014). This estimate was not applied for molecular clock calibration as the bison age is too recent.

2.7. Ancestral range reconstruction

We used S-DIVA, implemented as options within RASP software (Yu et al., 2015) to reconstruct the possible ancestral ranges of *Utricularia* species on the phylogenetic tree, based on concatenated sequences (total evidence) generated with Bayesian inference. For that, the areas of occurrence were set as nine regions according to Taylor (1989): (A) North America; (B) Central America; (C) South America; (D) Europe and North Africa; (E) North Asia; (F) Africa; (G) Tropical Asia; (H) Malaysia; and (I) Australia, New Caledonia, and New Zealand. For S-DIVA analysis the maximum areas was kept as four.

3. Results

3.1. Analyses of individual and combined datasets, congruence and phylogenetic relationships of infrageneric taxa

The six markers resulted in alignments of between 608 (*rbcL*) and 1257 (*trnL-trnF*) sites, and the combined dataset (all the six markers concatenated) resulted in 6226 sites (Table 3). Since the preliminary alignments of ITS sequences resulted in some sequences with less than 60% pairwise similarity, we chose to exclude the dissimilar sequences to minimize the noise. Thus, with the aim to identify misaligned sequences caused by saturation and with lower level of identity (pairwise similarity < 65%), we applied the MaxAlign (Gouveia-Oliveira et al.,

Table 3
Matrices and statistical analyses of alignments and cladograms inferred by maximum parsimony (boot = bootstrap; CI, RI = consistency index and retention index, respectively, based on maximum parsimonious trees; ML = maximum likelihood; MP = maximum parsimony; MPT = most parsimonious trees; PP = posterior probabilities based on Bayesian approach).

Matrix	Genome	Terminals (*n*)	Sites considering gaps (bp)	Variable sites (pb (%))	Parsimonious informative sites (pb)	Clades with support ≥ 50% (*n* (%))[a]			CI	RI
						Boot MP	Boot ML	PP		
rbcL	Chloroplast	153	608	128 (21)	101	40 (26)	46 (30)	54 (36)	0.53	0.92
matK	Chloroplast	119	960	582 (61)	471	63 (53)	70 (59)	72 (61)	0.57	0.89
rpl20-rps12	Chloroplast	79	1096	498 (45)	345	35 (45)	45 (58)	50 (64)	0.65	0.90
rps16	Chloroplast	68	1220	739 (61)	503	49 (73)	62 (93)	64 (96)	0.48	0.59
trnL-trnF	Chloroplast	73	1257	736 (59)	505	55 (76)	64 (88)	62 (86)	0.47	0.68
ITS	Nucleus	78	1091	818 (75)	753	34 (44)	39 (51)	50 (65)	0.43	0.83
Combined[b]	Chloroplast + Nucleus	93	6226	3449 (55)	2442	69 (75)	88 (96)	86 (93)	0.40	0.62

[a] The percentage of clades (%) was calculated from the total number of possible clades of the trees (= total OTUs-1).
[b] Only one sequence per species was used. Only taxa with at least two markers were considered for the combined dataset.

2007) to the ITS alignment. After running the analysis, 27 sequences were excluded from the original ITS dataset: *Pinguicula crystallina* (DQ438082.1), *P. filifolia* (AB212104.1). *P. lusitanica* (DQ222960.1), *P. primuliflora* (DQ222964.1), *P. vulgaris* (DQ438093.1), *Genlisea hispidula* (AB212112.1), *G. lobata* (AB212113.1), *G. pallida* (AB212114.1), *G. repens* (AB212116.1), *G. violacea* (AB212116.1), *Utricularia alpina* (AB212117.1), *U. bifida* (KF016005.1), *U. olivacea*, *U. furcellata* (KF016006.1), *U. intermedia* (DQ225109.1), *U. laxa*, *U. meyeri*, *U. minor* (AB212118.1), *U. multifida*, *U. nana*, *U. nelumbifolia*, *U. neottioides*, *U. purpurea*, *U. reflexa*, *U. reniformis* (DQ225108.1), *U. striata*, and *U. striatula* (KF016004.1). After that, the ITS dataset (ITS1 + 5.8S + ITS2) comprised 1091 aligned nucleotides. The decision to exclude those 27 sequences decreased the proportion of variable sites, and this decision improved the robustness of the phylogenetic analyses by minimizing the homoplasy from the original dataset (data not shown).

Even after the exclusion of the sequences with low pairwise similarity, the ITS dataset contained a relatively high number of variable sites (75%), followed by *matK* and *rps16* from cpDNA (61% each; Table 3). Despite the high level of substitutions found in ITS sequences, this dataset resulted in trees with an intermediate level of resolution, considering the proportion of clades with support of 50%, or more, for the six markers used (Table 3; Fig. 3). The *rps16* and *trnL-trnF* datasets resulted in the highest proportions of supported clades, unlike *rbcL* that showed the lowest proportion of clades with high support, with 26% of clades supported by parsimony, 30% supported by maximum likelihood, and 36% of clades supported by Bayesian posterior probabilities (with values of 50%, or more). Nevertheless, the isolated *rps16*, *trnL-trnF* and the ITS datasets resulted in the lowest consistency indices (CIs = 0.48, 0.47 and 0.43, respectively; Table 3). The *rbcL*, *matK*, and *rpl20-rps12* datasets resulted in the highest retention indices (0.92, 0.89, and 0.90, respectively).

According to the phylogenetic hypotheses based on the combined datasets (Fig. 4) and on each isolated sequence (supplementary material), general topologies were congruent and supported most sections of the infrageneric classification of the genus *Utricularia* as proposed by Taylor (1989).

The combined tree also supports *Utricularia* subgen. *Polypompholyx* as the sister group of the clade comprising subgenera *Utricularia* + *Bivalvaria* (maximum parsimony bootstrap [MP], maximum likelihood bootstrap [ML], and posterior probability [PP] = 1; Fig. 4) and thus corroborating with previous studies (Jobson and Albert, 2002; Jobson et al., 2003). The individual datasets, *matK*, *rps16*, and *trnL-trnF* also support this topology. According to Bayesian estimates, *rbcL* and ITS sequences do not resolve the relationship between the three subgenera resulting in a polytomy, and *rpl20-rps12* places subgen. *Polypompholyx* nested within subgen. *Bivalvaria* (supplementary material).

3.2. Evolution of life forms in Utricularia

The ancestral life form of *Utricularia* is terrestrial, according to the isolated markers and also the combined dataset (Fig. 4) corroborating with what has been proposed by Müller and Borsch (2005) and Müller et al. (2006). This plesiomorphic condition is in agreement with the predominant life form of the sister genus *Genlisea* (Fischer et al., 2000; Fleischmann et al., 2011) and *Pinguicula* (Casper, 1966; Roccia et al., 2016). Most aquatic species are nested in *Utricularia* sect. *Utricularia*, and this life form arose at least four times in the genus *Utricularia* according to our hypothesis: (1) *U. volubilis* (sect. *Pleiochasia*; also, *U. tubulata* is an aquatic species of this section, but it was not included in this study), (2) *U. foveolata* and *U. uliginosa* (sect. *Oligocista*, assuming one synapomorphy with the reversion to *U. graminifolia*, or a synapomorphy to the *U. foveolata-U. graminifolia-U. uliginosa* clade, since this species can be considered aquatic according to Taylor, 1989), (3) *U. nelumbifolia* (sect. *Iperua*, an aquatic and epiphyte species found inside bromeliads phytotelmata), and (4) the clade comprising sect. *Utricularia* + sect. *Vesiculina* + sect. *Avesicaria* + sect. *Setiscapella* + sect. *Nelipus*. In this latter clade, it is necessary to assume the reversion to *U. flaccida* and species from sect. *Setiscapella*, and also the arisen rheophytic life form of sect. *Avesicaria* (*U. neottioides* and *U. oliveriana*). The ephiphytic form occurs as a synapomorphy in *U. alpina* and *U. asplundii* (or a synapomorphy to the group formed by the sections *Iperua* and *Orchidioides*, with possible reversions to the species with other life forms), and also of *U. striatula*; in the latter case as a possible synapomorphy to sect. *Phyllaria* since this section is formed mostly by lithophytes and also epiphytes.

3.3. Biogeography and ancestral ranges reconstruction

The molecular clock tree based on *rbcL* sequences is shown in Fig. 7, and the divergence time estimates for the major lineages are depicted in Table 4. According to our estimates, the last common ancestor of *Genlisea* + *Utricularia* was possibly a South American lineage (Fig. 6) that arose 39 million years ago (mya). The genus *Utricularia* diverged around 30 mya and dispersed to Australia, with the lineage represented by the subgenus *Polypompholyx*, and possibly to Africa afterwards (Figs. 6–8).

4. Discussion

4.1. Evolutionary and phylogenetic characteristics of different DNA sequences

This study used both chloroplast and nuclear DNA, targeting sequences with distinct functions in the genome (*rps16* intron, *rpl20-*

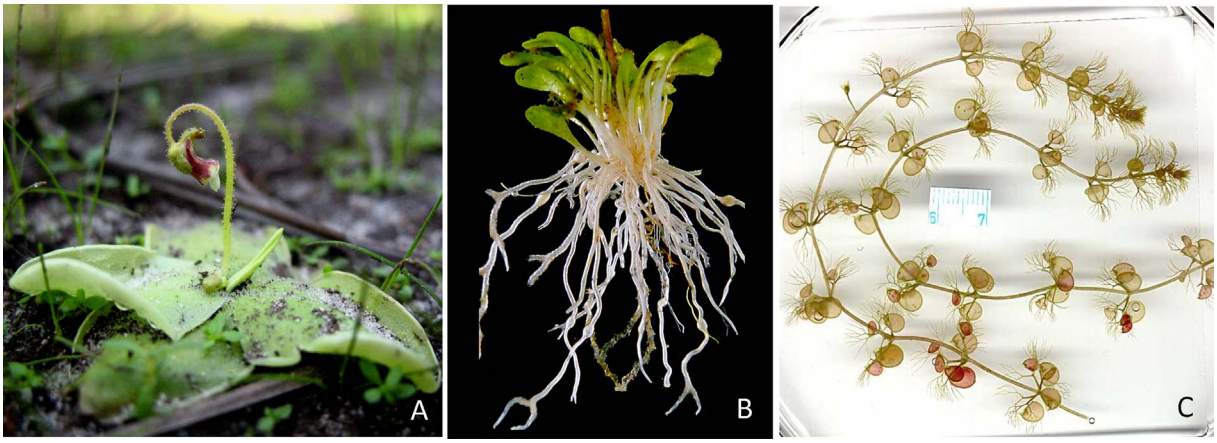

Fig. 1. Habit of the three genera: (A) *Pinguicula*, (B) *Genlisea* and (C) *Utricularia*.

Fig. 2. *Utricularia* species: (A, L) *U. reniformis* A.St.-Hil.; (B) *U. pubescens* Sm.; (C) *U. neottioides* A.St.-Hil. & Girard; (D) *U. cucullata* A.St.-Hil. & Girard; (E) *U. foliosa* L.; (F, I) *U. flaccida* A.DC.; (G) *U. laciniata* A.St.-Hil. & Girard; (H) *U. dichotoma* Labill.; (J, K) *U. geminiloba* Benj. (B – Photo credit to Gabriel Sabino).

rps12, *trnL-trnF*, ITS1, and ITS2 spacers, encoding genes *rbcL*, *matK*, and structural gene 5.8S). This allowed us to evaluate the different intrinsic traits of each marker due to their nature and evolutionary history. The *rbcL* gene showed the lowest proportion of variable sites, and its conservation (Palmer et al., 1988; Shivakumar et al., 2016) is given to the metabolic significance as ribulose bisphosphate carboxylase-oxygenase (Rubisco) as the core autotrophic carboxylase in oxygenic photosynthetic organisms (Raven, 2013). In contrast, ITS showed the highest proportion of variable sites, mostly resulting from the ITS1 and ITS2 spacers; the former with more variable sites (including indels) than the later. The ITS had the lowest consistency index (0.43) to this dataset (Table 3), due to the high concentration of homoplasies, but still contained a phylogenetically useful signal.

The *rps16* and *trnL-trnF* datasets had the most highly supported (> 50%) clades in trees (Fig. 3), and was therefore important for providing a signal for the phylogenetic hypothesis. In spite of *rbcL* having

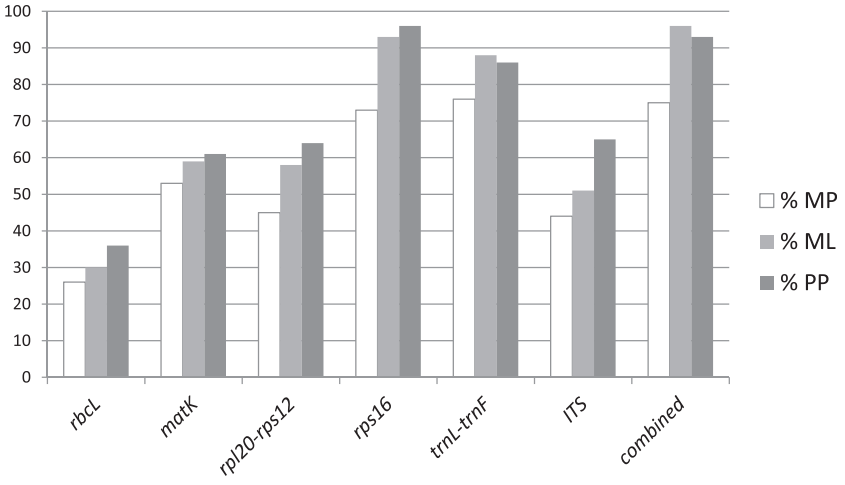

Fig. 3. Proportion of supported clades based on maximum parsimony, maximum likelihood, and Bayesian approaches to each DNA dataset (see also Table 3).

the highest retention index (0.92; Table 3), as a result of most variable sites supporting as synapomorphies, this dataset also had the lowest proportion of supported clades (Table 3; Fig. 3), and this result is evident in the trees based on maximum parsimony, maximum likelihood, and Bayesian approaches (Fig. 5). The *rps16* intron, with the combined datasets, resulted in the highest proportions of supported clades (Table 3; Fig. 3), corroborating the use of this intron as an important marker for phylogenies (Downie and Katz-Downie, 1999; Jobson and Albert, 2002; Jobson et al., 2003).

4.2. Phylogenetic relationships of major clades within Lentibulariaceae

The three genera of Lentibulariaceae, *Pinguicula*, *Genlisea*, and *Utricularia* are well established regarding the both taxonomic and phylogenetic approaches. The three genera are monophyletic, strongly supported by different sources of data, both morphological and molecular (Jobson and Albert, 2002; Jobson et al., 2003; Müller et al., 2004). *Pinguicula* comprises species with a common archetypal body organization of angiosperms, with vegetative organs including usually a short stem with helicoidally arranged leaves, and roots (Casper, 1966; Roccia et al., 2016). On the other hand, *Genlisea* and *Utricularia* have concentrated drastic evolutionary changes in body organization, with a deviation from the classical root-shoot (CRS) architectural rules or bauplan (Rutishauser, 2016) typically found in seed plants.

The Lentibulariaceae are carnivorous plants that commonly grow in nutrient-poor habitats (Adamec, 1997; Juniper et al., 1989). *Pinguicula* species have flypaper traps, with leaves covered by glandular trichomes (Casper, 1966; Heslop-Harrison, 2004). *Genlisea* species have leaves arranged in rosettes with heterophyllous leaves: photosynthetic and achlorophyllous leaves. These latter are specialized structures called rhizophylls that show positive geotropism (Fleischmann, 2012a). The rhizophylls are subterranean Y-shaped and tubular leaves, acting to fix the plants in the soil and also to trap small organisms (Płachno et al., 2008) as a source of nutrients (Barthlott et al., 1998). *Utricularia* species also possess foliar traps, which are called utricles. These are hollow bladders, usually 1–6 mm long with elastic walls and with mobile trap door (Vincent et al., 2011). *Utricularia* and *Genlisea* are the most divergent lineages within Lentibulariaceae, and they form a well-supported sub-clade sister to the *Pinguicula* clade (Jobson and Albert, 2002; Jobson et al., 2003; Müller et al., 2004; Fig.4).

4.3. Phylogenetic relationships within Utricularia

Here we show that subgenus *Polypompholyx* is the sister group to the clade formed by subgen. *Utricularia* and *Bivalvaria* according to the combined sequences (Fig. 4) and also individual *matK*, *rps16* intron and *trnL-F* datasets (supplementary material). According to *rbcL, rpl20-rps12* and ITS datasets (supplementary material), our phylogenetic reconstruction places the three subgenera in an unresolved polytomy. Previous studies based on *trnL-F* spacer, *rps16* intron (Jobson and Albert, 2002; Jobson et al., 2003; Reut and Jobson, 2010), and on *matK* (Silva et al., 2016) also support this tree topology (*Polypompholyx*, (*Bivalvaria*, *Utricularia*)); although Müller and Borsch (2005) had placed subgen. *Polypompholyx* as a sister group to subgen. *Bivalvaria*, and both as sister group to subgen. *Utricularia*. The incongruence found between studies may be due to taxonomic sampling, considering that these previous studies sampled no > 50 species of *Utricularia*, some of them with less than 15% of the *Utricularia* species.

According to our hypotheses based on combined and isolated DNA datasets, subgenus *Polypompholyx* is monophyletic and comprises the two clades of sect. *Pleiochasia* and sect. *Polypompholyx*; which supports Reut and Jobson (2010). Considering the pollen, Lobreau-Callen et al. (1999) recognized shared characters of both sections (classified as pollen type 1) and Taylor (1989) also considered this mostly tricolporate pollen type, present in sect. *Polypompholyx*, *Tridentaria*, and *Pleiochasia*, as the most primitive within the genus.

Section *Benjaminia* is monotypic and comprises the single South American species *Utricularia nana*. Phylogenetic studies reveal the incongruence of its phylogenetic position. Jobson et al. (2003) state that sect. *Oligocista* is paraphyletic, since it includes sect. *Benjaminia*. However, Müller and Borsch (2005) and Müller et al. (2006) positioned the group as sister to *Oligocista* + *Avesicarioides*. The results presented in this study, corroborated for all single and combined DNA datasets and with high support, support the paraphyly of sect. *Oligocista*; with sect. *Stomoisia* as sister group of the clade formed by the sections *Benjaminia* and *Oligocista*. Furthermore, Komiya (1973) proposed a new subgenus *Simplicirostra* containing sections *Orchidioides*, *Stomoisia*, *Avesicarioides*, and *Setiscapella*, based mainly on characters related to life form, utricles, and the morphology of reproductive structures (inflorescences, flowers and bracts/bracteoles). Although it is evident that there is no close relationship with sect. *Orchidioides* and/or *Setiscapella*, in the analysis based on *matK,* sect. *Oligocista* is revealed as paraphyletic too (also verified by Jobson et al., 2003), since sect. *Avesicarioides*, represented in this study by *U. rigida* (type species for the section), is nested within *Oligocista*. Taylor (1989) suggested the close relationship between sections *Oligocista* and *Benjaminia*, but justified their separation based on the morphology of the calyx lobes and utricles (shape and the lack of oral appendages). Here we propose combining both sections, as a single section, applying the oldest available name, sect. *Oligocista*.

The results presented here corroborate the inclusion of *Utricularia pubescens* (Fig. 1) in sect. *Calpidisca*. Taylor (1986) proposed the monotypic section *Lloydia* exclusively to accommodate this polymorphic species which is widely distributed in tropical America and Africa. It has diagnostic features, such as the peculiar peltate leaves (a

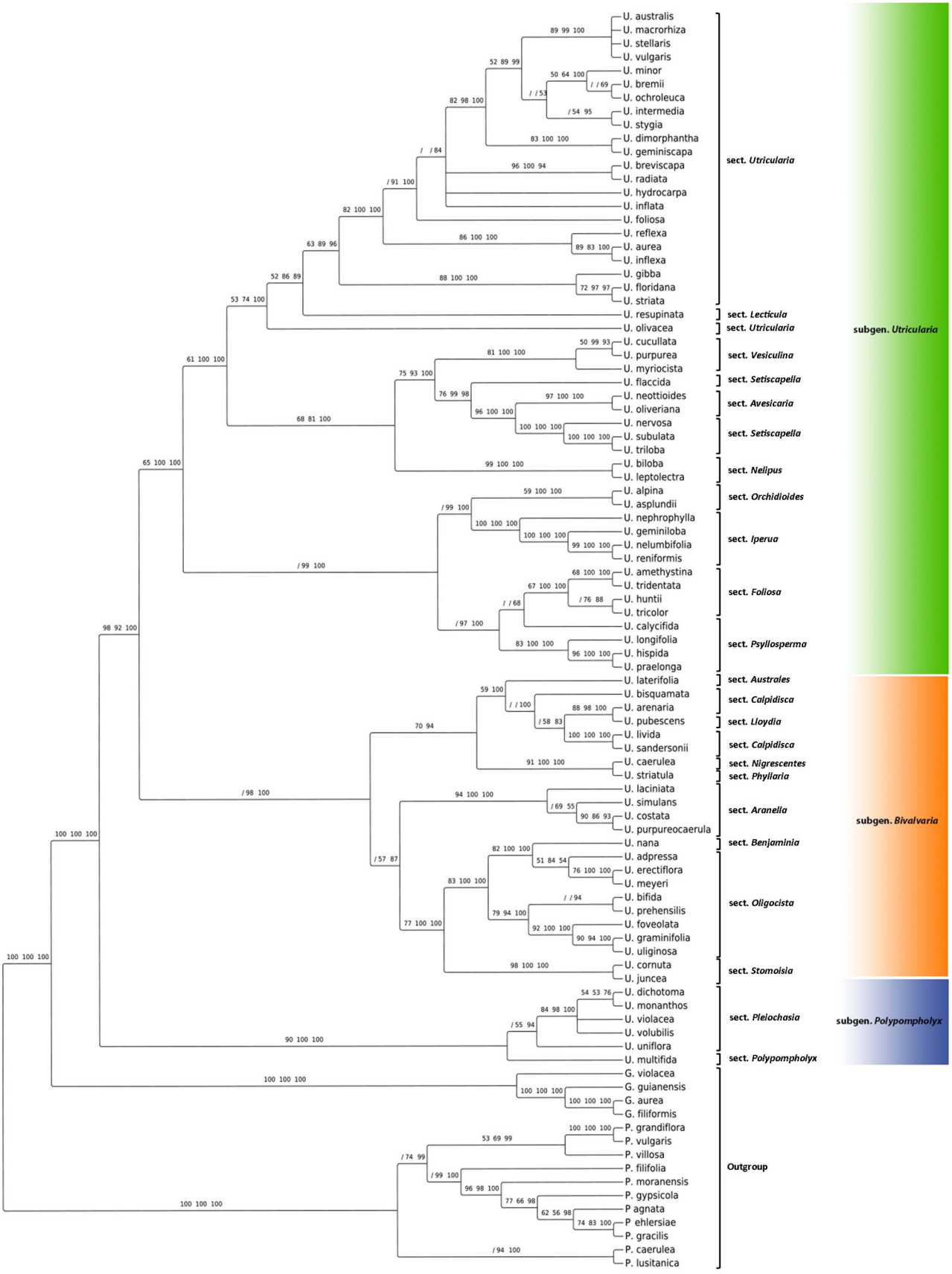

Fig. 4. Bayesian inference for concatenated sequences (*rbcL* + *matK* + *rpl20-rps12* + *rps16* + *trnL-F* + ITS). Numbers above the branches are the maximum parsimony bootstraps, maximum likelihood bootstraps and posterior probabilities. (/denotes branches with a support value < 50; U. = *Utricularia*; G. = *Genlisea*; P. = *Pinguicula*).

Table 4
Divergence times estimation (Mya) for selected nodes of *Utricularia*. (see also Fig. 7).

Node	Age according to this study (mya; 95% HPD)	Age according to Ibarra-Laclette et al. (2013)
Gen. *Pinguicula*	16.39 (7.27–27.74)	9.87
Genlisea-Utricularia clade	39.01 (21.05–59.95)	36.00
Gen. *Utricularia*	30.35 (17.12–45.86)	31.01
subg. *Polypompholyx* and *Bivalvaria*	26.94 (14.80–41.39)	–
subg. *Polypompholyx*	17.62 (6.53–30.20)	15.45
subg. *Bivalvaria*	24.98 (13.17–38.52)	27.20
subg. *Utricularia*	26.60 (15.13–39.97)	25.63
sect. *Utricularia*	12.76 (8.15–18.99)	11.11

very rare trait in the genus, present only also in *U. nelumbifolia*), and the morphology of the bracts, bracteoles and seeds. However Taylor (1989) also mentions that all other morphological characteristics of the plant, particularly the morphology of the utricles, support its inclusion in sect. *Calpidisca*, which is corroborated by our results based on molecular data. Thus, we suggest here that *U. pubescens* belongs in sect. *Calpidisca*.

Müller et al. (2006) argue that if they had sampled more species of sect. *Aranella* in their phylogenetic analyses, then probably, the section would have been proven to be polyphyletic. However, data from this research indicate that sect. *Aranella* is monophyletic and, according to the combined analysis, the section is closely related to sections *Benjaminia*, *Oligocista* and *Stomoisia* (Fig. 4).

Our concatenated tree results indicate that sect. *Psyllosperma* is clearly paraphyletic, supporting the results of Müller and Borsch (2005). For the ITS region, the species of this section form a monophyletic group, however the clades have low support. For the combined and *mat*K datasets, sect. *Psyllosperma* forms a paraphyletic clade and *U. calycifida* (sect. *Psyllosperma*) is nested in the clade of sect. *Foliosa,* as also demonstrated by Jobson et al. (2003) and Müller and Borsch (2005). Taylor (1986) proposed sect. *Psyllosperma* as an apparently "natural" group, even considering the similarity in pollen and seeds (Menezes et al., 2014) between the species of both sections (*Psyllosperma* and *Foliosa*). Müller and Borsch (2005) suggested the expansion of sect. *Foliosa* to include the sect. *Psyllosperma* – a proposal that is totally supported by this study.

Sect. *Iperua* is paraphyletic with *Orchidioides* based on the *matK* dataset, as also indicated by previous phylogenies (Müller et al., 2004; Müller and Borsch, 2005; Rodrigues et al., 2017). This might suggest that sect. *Iperua* should be included in sect. *Orchidioides*. Yet, the combined analysis (Fig.4) did not show this paraphyly, however *U. humboldtii* was not sampled in this analysis. Thus, according to previous studies (Müller and Borsch, 2005; Rodrigues et al., 2017) and based on the *matK* dataset, sect. *Orchidioides* should be expanded to include the sect. *Iperua*, since both compose a monophyletic group.

Phylogenies retrieved to date suggest that sect. *Vesiculina* is closely related to sect. *Setiscapella*. However, for the data presented, sect. *Vesiculina* is sister to *Setiscapella* + *Avesicaria*, except for the ITS region where sect. *Vesiculina* has been revealed as sister to sect. *Foliosa*; however, this is only supported by Bayesian analysis. Sect. *Setiscapella* is paraphyletic according to *matK* region, as in Jobson et al. (2003), however this occurrence cannot be confirmed by other regions since *U. flaccida* was not sampled for all markers.

4.4. Relationships among species of section Utricularia

Sect. *Utricularia* is the largest section in number of species, formally with 34 species sensu Taylor (1989), but totaling 36 taxa with *Utricularia stygia* Thor and *U. tenuicaulis* Miki (Fleischmann 2012b). Here we sampled 23 species of sect. *Utricularia*, the largest sample ever represented when compared to previous phylogenetic studies. The section is sister to sect. *Lecticula*, corroborating Jobson et al. (2003) and, according to Taylor (1989), has common morphological features including trap morphology, the ovoid shape with two simple appendices beside the mouth, prismatic seeds with isodiametric testa and oblate, 9–13 colporate pollen. According to our analyses, sect. *Utricularia* is divided into two major clades, clade I with the species *U. gibba*, *U. floridana* and *U. striata,* and another clade II with the remaining species.

Utricularia gibba is a cosmopolitan suspended aquatic species with extreme morphological polymorphism. Taylor (1989) highlights the similar morphology to *U. floridana* and *U. striata*, except for the leaves and seeds. The different datasets support this close relationship with the three species as a monophyletic group. Clade II can be subdivided into two subclades, one of them represented by *U. aurea*, *U. inflexa* and *U. reflexa*, which is highly supported by the combined analysis (Fig. 4) and *rpl20-rps12* dataset.

Considering the isolated datasets, some incongruence was found in the groups formed by species *Utricularia australis*, *U. bremii*, *U. dimorphanta*, *U. geminiscapa*, *U. intermedia*, *U. macrorhiza*, *U. minor*, *U. ochroleuca*, *U. stellaris*, *U. stygia* and *U. vulgaris*. Based on the chloroplast spacers *trnT-trnL* and *trnS-trnQ*, Kameyama et al. (2005) considered that *U. australis* originated from an asymmetric hybridization event between *U. australis* f. *tenuicaulis* (=*U. tenuicaulis*) and *U. macrorhiza*; which remains plausible based on our chloroplast spacer datasets. Some European species, such as *U. australis*, *U. bremii*, *U. ochroleuca*, *U. stygia*, are sterile and no longer cross-pollinate (Beretta et al. 2014), their propagation is predominantly clonal, which probably reduces genetic variability which possibly leads to the polytomy observed between closely-related species.

According to the combined analysis (Fig. 4) and *rbcL* dataset, sect. *Utricularia* is not monophyletic due to the free-floating aquatic species *U. olivacea*. This American species is minute and also taxonomically curious plants with scarce information available (Beal and Quay, 1968; Trevisan and Moço, 2011). Moreover, the *matK* tree nested *U. olivacea* within sect. *Utricularia* (as also shown by Müller and Borsch (2005) and Silva et al. (2016)) and in the same clade with *U. gibba* and *U. floridana*. While the *rbcL* sequence was newly generated for this study, we used the *matK* sequence of *U. olivacea* from GenBank (and the same used by Müller and Borsch (2005) and Silva et al. (2016)) for our phylogenetic constructions. *U. olivacea* can be found in close association with other *Utricularia* species (Taylor, 1989) and also with *U. gibba* (personal observation). Thus, contamination by *U. gibba* in *U. olivacea* samples cannot be excluded and further detailed study is needed, including more plant samples and also DNA markers.

The *rbcL* partial paleosequence found in the rumen of a Holocene Yakutian bison nested within the *Utricularia* sect. *Utricularia* clade (Fig. 7, *U.* "bison") and a confident identification to which species this DNA sample might belong was not possible.

4.5. Evolution of Utricularia life forms

Bladderworts are usually found in wet soils in open areas, ponds and lakes, streams, and on rocks or tree trunks (Taylor, 1989). They colonize different environments, from land (with terrestrial, lithophyte and epiphyte species) to open water (with free-floating and affixed aquatic species, rheophytes, and epiphytes – in the latter case on tree trunks as *U. jamesoniana* and *U. quelchii* (sect. *Orchidioides*), or even as aquatic-epiphytes in bromeliad tanks, as is the case of *U. nelumbifolia* (Płachno et al., 2017), *U. humboldtii*, and *U. reniformis* (sect. *Iperua*; Taylor, 1989). Some species may occupy different environments and thus can be classified in different categories (*Utricularia alpina* can be terrestrial and epiphytic – e.g. Valdés, 2008; *U. reniformis* can be terrestrial, epiphytic or even lithophytic, and *U. gibba*, which is commonly aquatic and has also be found on emergent and submerged leaves of *Salvinia*, a common fern found in ponds, and thus can be considered as an epiphyte – personal observation). Although this broad ecological amplitude cannot properly be generalized for all *Utricularia* species, this can be a complicating factor in assessment and discrete categorization of the life

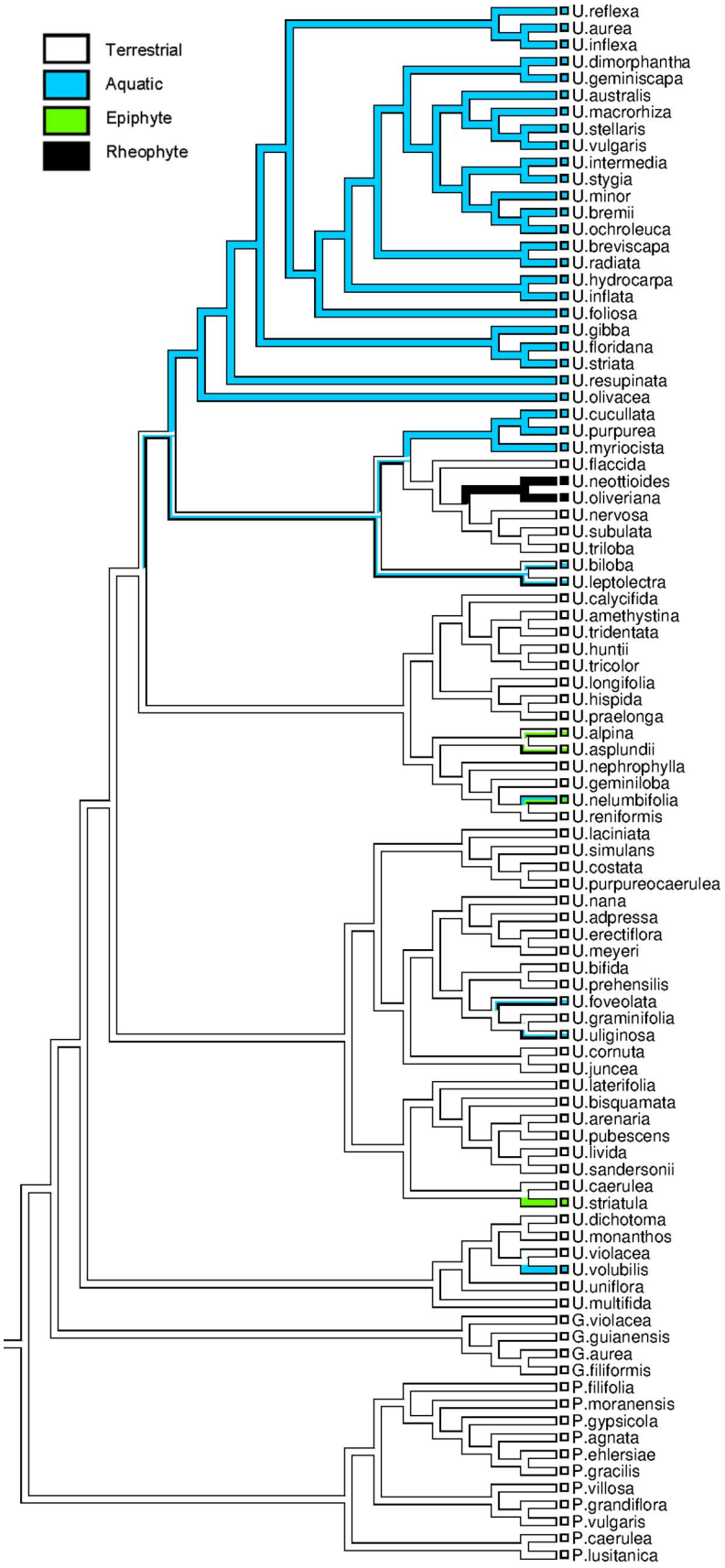

Fig. 5. Distribution of the life forms of Lentibulariaceae, with focus on *Utricularia* species, based on the Bayesian combined tree. (U. = *Utricularia*; G. = *Genlisea*; P. = *Pinguicula*).

forms. The vegetative organs of *Utricularia* species occasionally do not show a clear delimitation, so it can sometimes be a hard task to distinguish stem or stolon from leaves. Despite the stable bauplans (architectural body rules) found in floral organs and reproductive structures, the morphological ambiguity of vegetative organs and changes in their bauplans fit more properly to the continuum approach

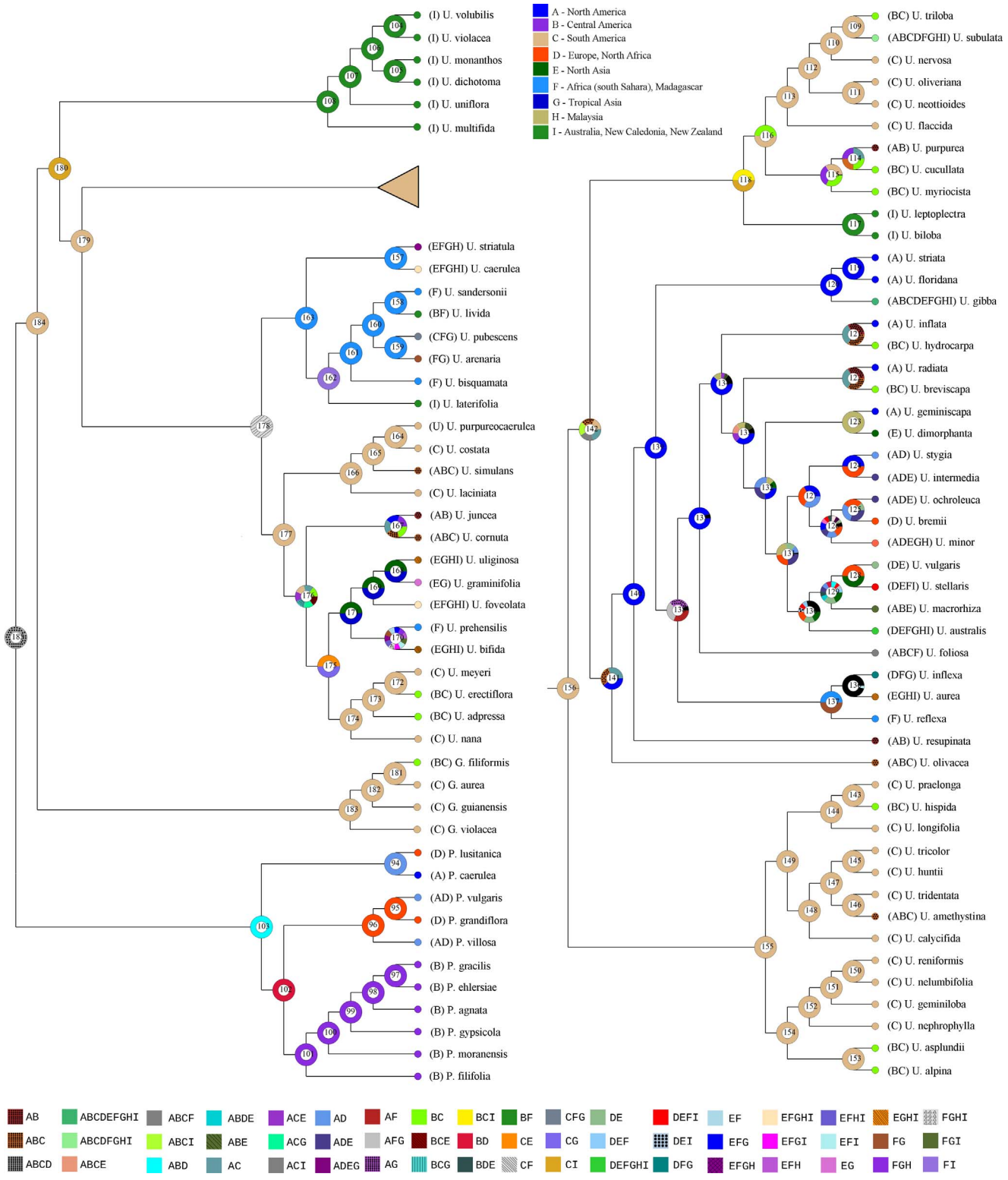

Fig. 6. Reconstruction of the possible ancestral ranges of *Utricularia* species on the phylogenetic tree based on concatenated sequences (total evidence) generated with Bayesian inference. The areas of occurrence were set as nine regions : (A) North America; (B) Central America; (C) South America; (D) Europe and North Africa; (E) North Asia; (F) Africa (south Sahara) and Madagascar; (G) Tropical Asia; (H) Malaysia; and (I) Australia, New Caledonia, and New Zealand.

and fuzzy "Arberian" morphology (Arber, 1950; Rutishauser, 1999; Rutishauser and Isler, 2001). The developmental switches and plasticity to unusual vegetative morphologies, comparable only to the Podostemaceae (river-weeds), has possibly facilitated the evolution of species diversity (Rutishauser, 2016) and an adaptation to and colonization of different habitats.

According to our phylogenetic hypothesis, and also supported by previous phylogeny studies (Jobson and Albert, 2002; Jobson et al., 2003; Müller et al., 2006), the terrestrial habit is plesiomorphic for the family Lentibulariaceae, as a common life form for most *Pinguicula* (Casper, 1966) and *Genlisea* species, with a few shifts to aquatic life form in *Genlisea* (e.g. *G. angolensis*, *G. guianensis*; Fleischmann, 2012a).

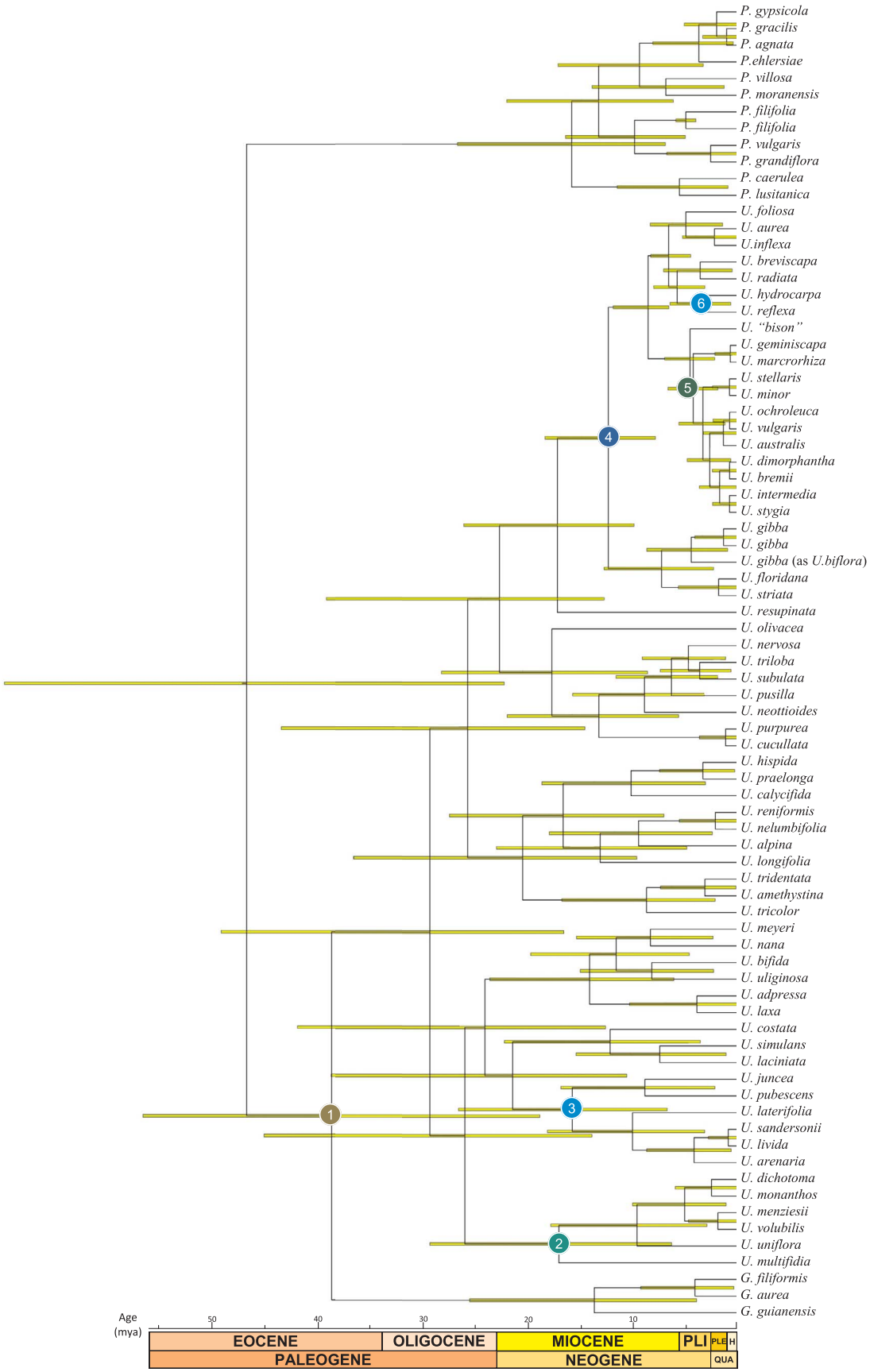

Fig. 7. Chronogram of Lentibulariaceae, with focus on *Utricularia* species, based on BEAST analysis using the *rbcL* dataset. Yellow bars indicate 95% highest posterior density intervals. Nodes of interest were marked as 1–6 (see also Fig. 8). (PLI = Pliocene; PLE = Pleistocene; H = Holocene.)

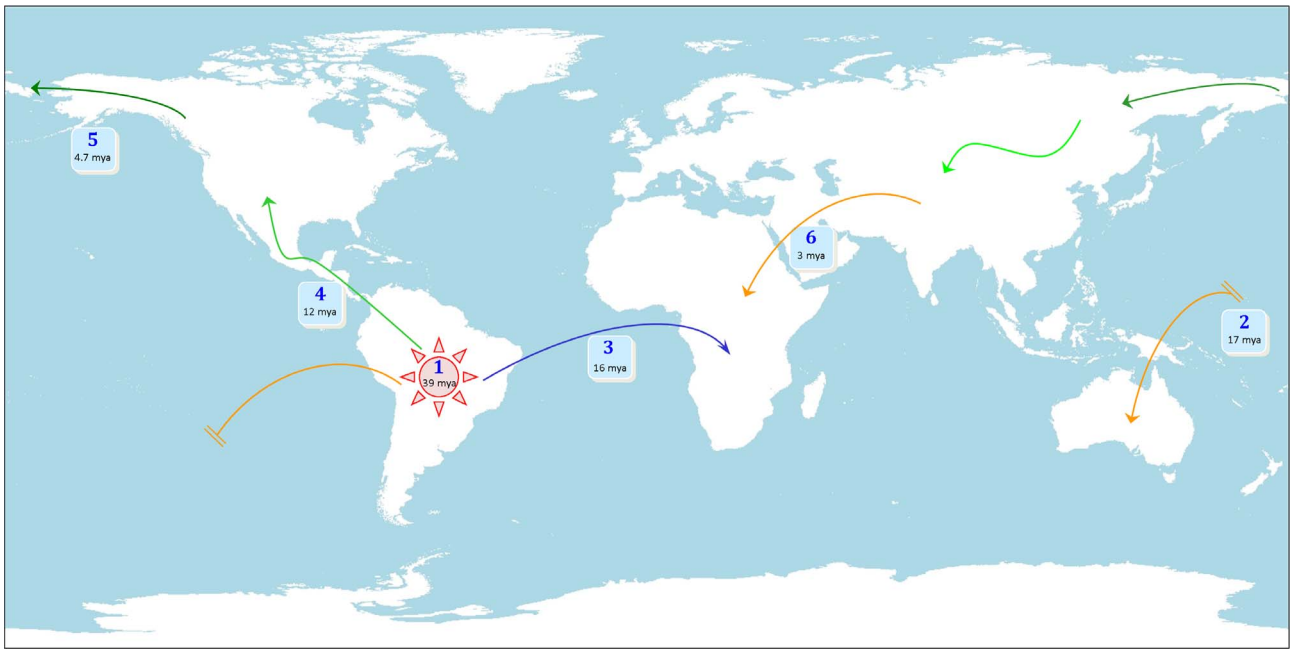

Fig. 8. Inferred dispersal routes of *Utricularia* lineages with dates based on the *rbcL* chronogram (Fig. 7). The boxes denote the nodes of interest, represented also in Fig. 7, with their respective possible ages.

For the *Genlisea* + *Utricularia* clade, this pattern was inherited and represents the most common life form for the various lineages of *Utricularia* – subgenera *Polypompholyx*, *Bivalvaria* and *Utricularia* (Guisande et al., 2007). Species with this pattern are usually found in peaty, sandy or even stony environments, but require high levels of humidity for their growth (Taylor, 1989).

Perhaps one of the most intriguing life forms is that of rheophytes. This habit is characterized by plants adapted to living in shallow rivers and streams (Font Quer, 1953). The shoots are strongly attached to rock surfaces by very specialized organs called appressoria that are lignified shoot projections which are responsible for anchoring the plants in even very rapid streams. This habit, studied extensively by Steenis (1978), is quite rare in *Utricularia* and is represented by a few species, including sect. *Avesicaria* (with the Neotropical species *U. neottioides* (Adamec et al., 2015) and *U. oliveriana*), *Avesicarioides* (with the African species *U. rigida* and *U. tetraloba*; the former represented in this study by *matK* sequence), sect. *Mirabiles* (*U. heterochroma* and *U. mirabilis*), and sect. *Choristothecae* (*U. choristotheca* and *U. determannii*). From a phylogenetic perspective, the rheophytic habit appeared at least twice in the evolutionary history of *Utricularia* (Müller and Borsch, 2005): once in the lineage of the African sect. *Avesicarioides* (*U. rigida* and *U. tetraloba*) and the other in the South American sect. *Avesicaria* (*U. neottioides* and *U. oliveriana*; Fig. 5). Thus, it is possible to see two homoplastic processes of parallel evolution when both rheophytic origins have the terrestrial life form as ancestral conditions. The epiphytic form also evolved at least twice from terrestrial species in the sections *Iperua* and *Orchidioides* (Fig. 5).

The aquatic life form is the second most common of all *Utricularia* species –the terrestrial form is the most common (Guisande et al., 2007; Taylor, 1989). Most aquatic species are from sect. *Utricularia*, which presents different subtypes of this life form (freely floating and affixed forms, living near the water surface commonly as amphibious or even semi-terrestrial or in a deeper water column). According to our phylogenetic hypotheses, the aquatic life form was derived from the terrestrial one and evolutionary shifts might have occurred more than once within the genus *Utricularia* (Fig.5). This scenario supports the hypothesis of multiple colonization of the aquatic environment, which should have been easy given the phenotypic plasticity of *Utricularia* and the fact that the traps work in water (Juniper et al., 1989; Taylor, 1989).

4.6. Phylobiogeography of Utricularia

Utricularia is a cosmopolitan genus distributed in all continents, except within the Antarctic Circle, most deserts, and most oceanic islands. Their presence on remote, oceanic islands, such as Galapagos and Hawaii, is possibly due to an accidental introduction (Taylor, 1989). The boreal boundary for the genus is represented by *U. intermedia*, *U. minor*, and *U. vulgaris* in the Arctic Circle (transgressing 70° N). The southern boundary is represented by *U. monanthos* that extends to near 47° S in wet soils and streams with shallow water in New Zealand (Taylor, 1989).

The genus *Utricularia* is represented by around 240 species and the species-richest regions occur mainly in tropical zones, with most taxa concentrated in South America (around 80 species, mainly in Southeast to Northeast Brazil and the Guyana Shield), and Australia, New Zealand and Central America, represented by around 70 species, and Tropical Asia, with around 50 species (McPherson, 2010; Taylor, 1989). South America and Australia are also the regions with the highest concentration of endemic species (76% of the Australian and 65% of the South American *Utricularia* species are endemic). The African continent has the third highest species richness (around 40 species), with taxa distributed throughout the continent (except the Sahara Desert) and Madagascar (McPherson, 2010; Taylor, 1989).

Studies on biogeography including chronologies of *Utricularia* are notably scarce, mainly due to a lack of fossils for confident calibrations. Ibarra-Laclette et al. (2013) inferred a molecular clock estimate based on *trnL*, *rps16*, *coxI* and *maK* sequences and applied as a calibration point the divergence time of the common ancestor of *Pinguicula* and *Utricularia-Genlisea* clade, which was estimated at 42 million years ago (mya) based on a relaxed molecular clock (Bell et al., 2010). Here we propose a molecular clock tree based on *rbcL* sequences and calibrated by using two references: (i) fossilised pollen of *U. minor* (dated between 11.6–23.0 mya; Muller, 1984) and (ii) the insular separation of the island Isla de la Juventud from the Western portion (Pinar del Río province) of Cuba (1.8–5.3 mya) and resulting divergence of *Pinguicula filifolia* lineages (Ferrera et al., 1990). The chronological tree presented here (Fig. 7) resulted in similar divergence times in general when

compared to Ibarra-Laclette et al. (2013). The time estimates for each clade throughout the discussion are from our present study and also from Ibarra-Laclette et al. (2013; both values in parentheses in mya).

According to our results (also suggested by Jobson et al., 2003 and Fleischmann et al., 2011), the last common ancestor of *Genlisea-Utricularia* clade was possibly a South American lineage, that arose 39 mya (36 mya estimated by Ibarra-Laclette et al., 2013) (Figs. 6–8). *Utricularia* diverged from its sister genus 30 mya (31 mya according to Ibarra-Laclette et al., 2013) and dispersed to Australia with the lineage represented by subgenus *Polypompholyx* (17 mya by our estimate and 15 by Ibarra-Laclette et al., 2013) and possibly to Africa afterwards (16 mya; 21 mya by Ibarra-Laclette et al., 2013). Probably other transcontinental dispersals may have occurred, one of them represented by sect. *Nelipus* that includes three Australian, Tropical Asian and Malaysian species (*U. biloba*, *U. limosa*, and *U. leptoplectra*).

The dispersal to North America possibly occurred from South America, between 11 mya (Ibarra-Laclette et al., 2013) and 12 mya (this study; Figs. 6–8), in the middle of Miocene, even with the land discontinuity between North and South America as a reason of the absence of Isthmus of Panama that arose some million years later in Pliocene around 2.8 mya (O'Dea et al., 2016). North America has been colonized by species that are widespread also in Central and South America, such as *U. amethystina*, *U. cornuta*, *U. juncea*, *U. olivacea*, *U. purpurea* and *U. resupinata* (Taylor, 1989).

The colonization of *Utricularia* into Eurasia probably occurred via the Bering Strait (DeChaine, 2008) to Northwestern Asia and also to Europe at around 4.7 mya by long-distance dispersal (Figs. 6–8), and this hypothesis can be corroborated considering the occurrence of hibernacula in *U. minor* in tropical Alpine Papua New Guinea, probably distributed by migratory birds (Taylor 1977). Also, the hypothesis of trans-Atlantic dispersal cannot be excluded (Abbott and Brochmann, 2003). The presence of *U. intermedia*, *U. macrorhiza* and *U. ochroleuca* in North American and Eurasian floras (Taylor, 1989) may corroborate the hypothesis of an American origin of Eurasian lineages responsible for spreading through Europe and southern Asia. The presence of *U. aurea*, *U. inflexa* and *U. reflexa* nested within sect. *Utricularia* is particularly intriguing, although they have been classified in this section (Taylor, 1989). *Utricularia reflexa* is a highly variable suspended aquatic species restricted to Africa which, together with other species from this section, represents a group mostly composed of species from Americas and Europe. According to our estimate (Figs. 6 and 7), the lineage of *U. aurea, U. inflexa* and *U. reflexa* possibly reached the African continent from Tropical Asia very recently, around 3 mya, which would be strongly supported if *U. corneliana* (Jobson, 2012) was indeed *U. reflexa* (Fleischmann, 2015).

The extensive geographic range of the genus and also of some species such as *Utricularia gibba* and *U. subulata,* which have a pantropical distribution (Taylor, 1989), may be at least partly due to long dispersal by migratory birds, facilitated by the morphology of seeds – most species of *Utricularia* have diminutive dust-like seeds (Eriksson and Kainulainen, 2011; Menezes et al., 2014) with a very reduced embryo (Płachno and Świątek, 2010).

The very small seeds in several species contain a foveolate testa or projections formed by periclinal walls (e.g., see *U. reniformis* – Płachno et al., 2009). These traits, when combined with the small seed size, are favorable for the formation of air bubbles, thereby providing buoyancy and aiding in seed dispersal (Menezes et al., 2014). Many species of *Utricularia* are found in bogs and hydromorphic soils, usually associated with seasonal water bodies or streams arising from rain – habitats very favorable for water dispersion. Also, wind dispersal may be the main process for species as *U. humboldtii*, *U. nelumbifolia*, and *U. reniformis* (Taylor, 1989).

Vegetative propagules, known as winter buds or turions are present in 12 temperate or subtropical species, are dormant overwintering storage organs formed by modified shoot apices with reduced leaves, rich in starch or proteins (Płachno et al., 2014). Turions resist dessication and may provide a means of long-distance disersal on the feet of water birds flying between lakes. Stolon fragmentation may also play an important role for species dispersal, a very common and efficient manner to rapidly colonize new water bodies. Stolon fragmentation of aquatic species (such as *U. cucullata*, *U. foliosa* – Fig. 2, *U. reflexa*) is very important for dispersal and also for colonization, especially if the habitats are seasonal water bodies created by rain water. Each small stolon fragment (which may be < 1 cm long, as is common for *U. gibba*) can produce a much-divided new plant in a few weeks. Dispersal and colonization using clonal reproduction are a rapid and efficient process for aquatic angiosperm reproduction and, when associated with the possible long-distance dispersal (Les et al., 2003), can at least partly explain the pantropical distribution of *U. gibba*. An extensive population study of *U. gibba* based on ortholog and paralog copies of rDNA ITS, which surveyed inter and intrapopulational individuals from Brazil and Cuba, showed that Cuban plants share haplotypes from individuals sampled in Northeast Brazil. These results support the rapid northward dispersion hypothesis of this species in the Americas, probably facilitated by clonal reproduction.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.10.010.

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