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DNA barcoding approach fails to discriminate Central European bladderworts (Utricularia, Lentibulariaceae), but provides insights concerning their evolution

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ABSTRACT

The main features to distinguish the seven native *Utricularia* species occurring in central Europe are found in flower shape, but being rarely flowering identification is often doubtful and uncertain. A recent morphometric work highlighted that there are no univocal reliable extra-floral morphological features allowing a safe identification at species level. Therefore, DNA barcoding approach is attempted here. Molecular analyses were performed to search for DNA barcodes using nuclear ITS (rDNA), plastid (cpDNA) *trn*L-*trn*F IGS and *rps*16 intron sequences. Generally, the barcoding approach failed to discriminate *Utricularia* species, although it could be of some help in the *U. minor* aggregate. With few exceptions, *U. bremii* shows peculiar DNA regions different from *U. minor* for both plastid markers investigated. However, interesting hypotheses could be derived from the obtained networks, including hybridization events to explain the rise of mostly sterile species, such as *U. stygia*. This species clusters with the other species of the *U. intermedia* aggregate in plastid phylogenetic graphs, while it is closely related to species of the *U. minor* aggregate in ITS phylogenetic graphs. Additionally to *U. stygia*, *U. ochroleuca* also shows some incongruences in the different markers, at least for some accessions, pointing to the possible occurrence of hybrids.

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Introduction

Since its original description (Hebert et al. 2003), the barcoding approach has gained more and more credit among taxonomists, yet only in relatively recent years it has been applied successfully to plants (Hollingsworth et al. 2009, 2011). Indeed, plants are characterized by massive polyploidization, hybridization, introgression, clonal and/or unusual sexual reproduction, which provide constant difficulties for both identification and phylogenetic reconstruction purposes. Generally, being maternally inherited in angiosperms (Tilney-Bassett 1978; Kuroiwa 1991), plastid markers are related to seed dispersal, but seeds usually cover much shorter distances than pollen (Ghazoul 2005). Therefore, plastid genes may provide an underestimation of gene flow (Naciri et al. 2012), which instead, when conspicuous, represents a fundamental requirement for discriminating species (Petit and Excoffier 2009). For these reasons, multilocus and multigenomic approaches are needed for making barcoding applicable to plants. The multilocus approach (Fazekas et al. 2008) considers the use of different plastid markers combined, together or in place of the standardized markers, such as *rbc*L and *mat*K (Hollingsworth et al. 2009, 2011; Sandionigi et al. 2012). On the other hand, the multigenomic approach considers both plastid and nuclear (mostly Internal Transcribed Spacers, rDNA ITS marker) genomes (China Plant BOL Group 2011), so as to enable the recognition of hybridization and other misleading events, such as introgression and incomplete lineage sorting after recent speciation. Despite these precautions, molecular results may provide an overestimation of the differences among taxa (or an underestimation of the variability within a single taxon) when only one or two sequences per taxon are taken into account. To reduce the risk of such drawbacks, more populations of each species are needed and, for each one, more individuals as possible have to be sampled (Zhang et al. 2010; Bergsten et al. 2012).

Particular attention in applying the barcoding technique should be paid in the case of plants showing high mutational rates and clonal reproduction, such as species of the genus *Utricularia* L. (bladderworts). Bladderworts also epitomize the peculiar so-called "relaxed morphology" (Rutishauser and Isler 2001): the organ circumscription of their body is hard to delineate and the phenotypic plasticity is high, depending on the growth substrate as well as on other environmental factors (Taylor 1989; Płachno and Adamec 2007). Despite being the richest and most widespread genus (at least 250 species occurring in five continents; Taylor 1989; Fleischmann 2012) of carnivorous plants, *Utricularia* is instead

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less represented in Europe, where only nine species occur. Two of them are restricted to south-western Europe (*U. subulata* L. and *U. gibba* L.) and are easily distinguished by the other European taxa, so that they do not provide any identification problem considering both flowers and vegetative parts. The remaining seven species (*U. australis* R.Br., *U. bremii* Heer, *U. intermedia* Dreves & Hayne, *U. minor* L., *U. ochroleuca* R. Hartm., *U. stygia* Thor, and *U. vulgaris* L.), all aquatic and mostly occurring in Central Europe, are instead much more taxonomically problematic. As these species are often found on threatened habitats and their circumscription is not well known yet, they are all considered of conservation interest by the IUCN (Bilz et al. 2011), though four of them are listed as *Data Deficient* (DD) because of the mentioned identification problems. From the systematic point of view, they can be subdivided into three aggregates (aggr. hereafter): (1) *U. intermedia* aggr., also including *U. ochroleuca* and *U. stygia*, (2) *U. minor* aggr., also including *U. bremii*, and (3) *U. vulgaris* aggr., also including *U. australis* (Astuti and Peruzzi 2018a). These aggregates are easy to circumscribe on morphological grounds, but species inside each complex are difficult to identify (Astuti and Peruzzi 2018a, and literature cited therein), and for *U. intermedia* and *U. bremii* only very recently a lectotypification and a 2nd step lectotypification have been provided, respectively (Astuti and Peruzzi 2018b). Most of the diagnostic morphological features rely on flowers (Thor 1988; Taylor 1989; Tassara 2002), but these species are rarely flowering. For this reason, Astuti and Peruzzi (2018a) tried a traditional and geometric morphometric approach to test if even vegetative parts may provide diagnostic characters for species discrimination, as reported in various articles (Thor 1988; Taylor 1989; Moeslund et al. 1990; Tassara 2002; Schlosser 2003; Płachno and Adamec 2007; Gariboldi and Beretta 2008; Fleischmann and Schlauer 2014; Tison and de Foucault 2014). However, they demonstrated that shoot morphology, including features of quadrifid digestive glands, is not reliable and, in *U. minor* aggr., completely useless.

As regards the phylogenetic relationships of bladderworts, several papers have focused on Lentibulariaceae, and some sequences of *Utricularia* were also included (Jobson and Albert 2002; Jobson et al. 2003; Müller et al. 2004, 2006), whereas a few studies were properly targeted to phylogenetic reconstructions within the genus *Utricularia* (Müller and Borsch 2005; Silva et al. 2018). However, only Silva et al. (2018) performed their investigation on all central European species and using both plastid and nuclear markers, although each species was represented only by a single sequence. Within the concatenated tree built by merging nuclear and plastid sequences presented by Silva et al. (2018), central European species are subdivided into two clades, consistently with shoot morphology, including *U. australis* and *U. vulgaris* on one side, sister to all other species. The latter clade is in turn subdivided into two subclades: (1) one joining *U. bremii*, *U. minor*, and *U. ochroleuca*, with *U. bremii* and *U. ochroleuca* sister to each other and, together, sister to *U. minor*, and (2) the other one including *U. intermedia* and *U. stygia*. The closer relationship of *U. ochroleuca* with *U. minor* aggr., as compared with species of *U. intermedia* aggr., can be surprising based on morphological aspects, but a hybrid origin of *U. ochroleuca* and *U. stygia*, through a cross between *U. minor* and *U. intermedia*, was previously postulated, as well as several hybrids have been suggested to occur among European species (Neuman 1900; Lindberg 1921; Schlosser 2003; Płachno and Adamec 2007). Taylor (1989) assessed that putative hybrids are instead dysploid vegetative apomicts. However, at least for *U. australis*, a hybrid origin is plausible and supported by means of molecular analyses (Kameyama et al. 2005). Taken alone, plastid trees were generally not concordant concerning relationships between central European species and resolution was low for most of the markers used, with the exception of *rps*16 intron and *trn*L-*trn*F intergenic spacer (Silva et al. 2018, Supporting Information). Nevertheless, for both *rps16* and *trn*L-*trn*F, no sequences of *U. bremii*, *U. ochroleuca* and *U. stygia* were obtained (for *rps*16, no sequence of *U. minor* either). According to Müller and Borsch (2005), plastid haplotypes of *U. australis* are closer to *U. vulgaris* than to the putative parental species *U. macrorhiza* Leconte, partially consistent with the involvement of the latter species as male parental in the origin of *U. australis,* as hypothesised by Kameyama et al. (2005).

A fingerprinting approach for analysing phylogenetic relationships among aquatic species of *Utricularia* was provided by Rahman (2006, 2007), who found a close relationship between *U. australis* and *U. macrorhiza*, and between these latter species and *U. vulgaris*. Interestingly, *U. bremii* and *U. minor* did not cluster together, as expected by considering their extreme morphological affinity, but the latter was close to *U. gibba*, while the former to *U. intermedia*. However, the few sampled species, the high molecular rates of evolution within *Utricularia* and the putative massive presence of indels may considerably affect the clustering obtained with this DNA fingerprinting approach (Weising et al. 2005), also considering that the fingerprinting analysis does not necessarily reflect phylogenetic relationships, since it is impossible to establish the primary homology of the bands.

The aim of our study was to check whether reliable molecular tools for species identification exist, in order to provide a framework for the delimitation of the distribution range of each species, which is a necessary step for their conservation assessment. In connection with molecular analyses, we aimed also at reconstructing the phylogenetic relationships occurring among taxa, in order to infer their origin and circumscription.

Material and methods

Sampling

For all species, we sampled fresh shoots for at least two populations each, with the exception of *U. ochroleuca*. For this latter species, only cultivated plants (Collection of aquatic and wetland plants, Institute of Botany, Třeboň, Czech Republic), all originally collected from Třeboň area, were available, as this species is subject to strict conservation programmes in all countries of occurrence (e.g., Germany, France, Poland, Czech Republic, Finland, and Sweden).

Table 1. Populations sampled for molecular analysis and GenBank accessions.

Species	Population	Acronym	ITS	*trn*L-*trn*F IGS	*rps*16 intron
U. australis	Czech Republic, Třeboň Basin	AD	/	MH051617	MH051561
	Germany, Saxony-Anhalt, Oranienbaum Heide*	GEO	/	MH051618- MH051620	MH051562- MH051564
	Italy, Tuscany, Viareggio*	ITV	MH051675-MH051677	MH051621- MH051625	MH051565- MH051567
U. bremii	Czech Republic, Třeboň Basin	AD	MH051678	/	/
	Italy, Trentino-Alto Adige, Lake Monticolo*	IT	MH051679-MH051684	MH051626- MH051632	MH051568- MH051573
	Switzerland, Zurich, Katzensee*	SWK	MH051685-MH051686	MH051633- MH051635	MH051574- MH051576
U. intermedia	Czech Republic, Třeboň Basin	AD	/	MH051636	MH051577
	Russia, Leningrad Oblast, Lake Bezymannoyoe*	RUB	MH051687	MH051637- MH051640	MH051578- MH051580
	Russia, Leningrad Oblast, Lake Michurinskoye	RUM	MH051690- MH051692	MH051641- MH051643	MH051581- MH051583
U. minor	Czech Republic, Třeboň Basin	AD	MH051693	MH051644	MH051584
	Italy, Trentino-Alto Adige, Italy	ITT	MH051694- MH051697	MH051645- MH051649	MH051585- MH051590
	Russia, Leningrad Oblast, Lake Bezymannoye*	RUB	MH051698- MH051700	MH051650- MH051652	MH051591- MH051593
U. ochroleuca	Czech Republic, Třeboň Basin*	CZ	MH051701- MH051702	MH051654- MH051656	MH051595- MH051598
U. stygia	Czech Republic, Třeboň Basin	AD	/	MH051657	MH051599
	Italy, Trentino-Alto Adige, Lake Monticolo*	IT	MH051703- MH051706	MH051658- MH051664	MH051600- MH051606
	Switzerland, Zurich, Ambitzgi*	SWA	MH051707- MH051709	MH051665- MH051667	MH051607- MH051609
U. vulgaris	Czech Republic, South Moravia	AD	MH051710	MH051668	MH051610
	Russia, Leningrad Oblast, Lake Bezymannoye*	RUB	MH051711	MH051669- MH051671	MH051611- MH051613
	Russia, Leningrad Oblast, Lake Michurinskoye*	RUM	MH051712- MH051714	MH051672- MH051674	MH051614- MH051616

Populations already investigated morphologically by Astuti & Peruzzi (2018a) are marked by asterisks (*).

Most of the populations included in this study were already investigated morphologically by Astuti and Peruzzi (2018a). Only individuals safely identified, based on flower morphology, were used. See Table 1 for further details on sampling.

DNA extraction, amplification and sequencing

Genomic DNA was obtained from stolons, previously washed with distilled water in order to remove epiphytes and other particles, and stored in dry state in silica gel. In some cases, DNA extraction was performed following the protocol published by Lodhi et al. (1994, modified), but in most cases using Plant II DNA extraction kit (Machery-Nagel). In both cases, plant tissues were first macerated in a mortar with liquid nitrogen. Genomic DNA of samples labelled as AD (Table 1) were provided by LA.

Three markers, the plastid *trn*L-*trn*F intergenic spacer and *rps*16 intron and the nuclear ITS (Internal Transcribed Spacer) region (ITS1 + 5.8S + ITS2) were used for the analyses. Amplification of the three markers was performed using Taq DNA polymerase (Thermo Scientific Fermentas®, Pittsburgh, PA) and the primers and conditions listed in Table 2. Direct sequencing of PCR templates was carried out at GATC Biotech AG (Cologne, Germany), using an Applied Biosystems 3730xl Sanger sequencer. Obtained sequences of both strands (forward and reverse) were first assembled with the help of Chromas Lite v. 2.1.1 software (Australia Technelysium Pty Ltd.), to obtain a single sequence for each accession. Single sequences were aligned with Clustal X 2.1 (Larkin et al. 2007) and manually edited. Unfortunately, for *U. australis,* good quality ITS sequences were obtained for only one out of the two populations sampled.

DNA barcoding approach

The guidelines of Plant Working group of CBOL (http://www.barcoding.si.edu/plant_working_group.html) suggest the use of two plastid markers, *rbc*L and *mat*K genes. Unfortunately, *rbc*L has been found to be unhelpful for discriminating close species (China Plant BOL Group 2011), and when used in a wide sampling approach on *Utricularia* species, it was found highly conservative, and thus not suitable for barcoding (Silva et al. 2018). Hence, it is likely not effective on closely related taxa such as European species of *Utricularia*. On the other hand, according to Müller et al. (2004), *mat*K is very variable in *Utricularia*, showing the highest substitution rates found so far among angiosperms. Nevertheless, the *mat*K phylogenetic tree published by Silva et al. (2018, Supporting Information) showed that three different accessions of *U. minor* as well as two different accessions of *U. vulgaris*, respectively, do not cluster in the same clade, pointing to a potential failure in the barcoding approach. Moreover, Silva et al. (2018) were not able to obtain *mat*K sequences for *U. ochroleuca*, pointing to some difficulties in amplification and sequencing of this marker in this European species. For these reasons, we discarded these plastid markers, despite recommended as core-barcode for land plants (CBOL Plant Working Group 2009; Hollingsworth et al. 2011), in place of *trn*L-*trn*F IGS and *rps*16 intron. Jobson and Albert (2002) successfully used these latter markers, even if their study was exclusively aimed at the phylogenetic reconstruction of a wide range of *Utricularia* species, and not on the identification of a restricted group of closely related species. In addition, the availability of primers in the literature and the relative ease to obtain sequences, led us to choose these plastid markers for our studies.

Authors involved in CBOL Plant Working Group already proposed the use of ITS as a supplementary marker for barcoding (China Plant BOL Group 2011; Hollingsworth et al. 2011; Li et al. 2011). Being biparentally inherited, a nuclear marker may help to detect hybridogenic taxa when compared to plastid markers (Sang et al. 1995; Nieto Feliner and Rosselló 2007). For some of our target species, a hybrid origin has been hypothesized (Neuman 1900; Thor 1988; Schlosser 2003; Płachno and Adamec 2007), so that investigations on different genomic compartments (plastid and nuclear) may reveal conflicting tree or network topologies, pointing to hybridization events. Until now, ITS sequences

Table 2. Primers and PCR conditions.

	ITS (White et al. 1990)			*trn*L-*trn*F IGS (Taberlet et al. 1991)			*rps*16 intron (Oxelman et al. 1997)		
Conditions	Temperature	Time		Temperature	Time		Temperature	Time	
Initialization	95 °C	1′		94 °C	1′30″		94 °C	1′30″	
Denaturation	92 °C	30″		94 °C	1′	× 35	94 °C	30″	
Annealing	50 °C	50″	× 34	52 °C	1′		56 °C	30″	× 30
Elongation	70 °C	1′		72 °C	2′		72 °C	1′	
Final elongation	70 °C	10′		72 °C	15′		72 °C	15′	

were available only for four out of seven Central European species (Silva et al. 2018). For all these reasons, we decided to include ITS marker in our investigation.

Reconstruction of evolutionary relationships

A single phylogenetic tree may not be an appropriate representation of different incompatible phylogenetic signals, because evolution of organisms is also affected by hybridization, introgression and horizontal gene transfer (Linder and Rieseberg 2004). Moreover, reconstruction of relationships among taxa using gene (or non-coding DNA regions) markers, may not correspond to actual species phylogenies, because of misleading events such as gene loss and duplication, incomplete lineage sorting or recombination (Naciri and Linder 2015). A network may be more suitable for reconstructing evolutionary relationships (Huson et al. 2010) when reticulate evolution is likely to occur, and one way to represent conflicting signals in a network is the split graph. In the present study, networks were built using the Neighbor-Net function of SplitsTree 4 software (Huson 1998; Bryant and Moulton 2004). This approach is based on the distance method Neighbor Joining (NJ) (Saitou & Nei 1987), and splits are drawn by means of the Equal Angle method. Uncorrected p-distances were applied to the Neighbor-Net algorithm.

We also performed a phylogenetic analysis for both plastid and nuclear markers separately and combined in order to infer the phylogenetic relationships among target species. For this analysis, we built a phylogenetic tree under Bayesian inference using MrBayes software version 3.2.6 (Ronquist et al. 2012). Because of the presence of indels of different length and position among sequences, and hence potentially useful for barcoding, we performed phylogenetic analyses after gap coding. Gaps were coded using the MCIC setting of SeqState (Müller 2005) and the alignment was partitioned according to nucleotide and gap coding, respectively. $GTR+I+\Gamma$ model of nucleotide substitution was applied to the nucleotide partition, whereas a simple JC model was applied to gap coding partition. Analyses with gaps treated as missing were also performed. Posterior probability values (PP) were calculated with the Markov chain Monte Carlo method, using two runs each with four chains (one cold and three heated) started from a random tree, with parameters sampled every 500 generations. Once the standard deviation of split frequencies was below 0.01 (i.e. after 700,000 and 500,000 generations for plastid and ITS trees, respectively), calculation was stopped. Only trees subsequent to the burn-in (25%) were used to calculate a majority-rule consensus cladogram. For these analyses, *U. aurea* Lour. (GenBank AF482632 and AF482559 for *trn*L-F and *rps*16, respectively) + *U. inflata* Walter (GenBank AF488531 and AF488525 for *trn*L-F and *rps*16, respectively) and *U. dimorphantha* Makino (GenBank MG027749) + *U. aurea* (GenBank MG027742) + *U. inflexa* Forssk. (GenBank MG027743) were used as outgroup for cpDNA and rDNA, respectively. The outgroup choice was made by selecting those sequences falling just outside the clades containing our target species in the phylogenetic trees published in the Supporting Information by Silva et al. (2018). Accessions of *U. macrorhiza* (GenBank AF482657 and AF482581 for *trn*L-F and *rps*16, respectively; GenBank MG027747 for ITS) were also included in both trees.

Results

DNA barcoding

Sequence length ranged from 817 to 825 bp for *rps*16 intron and from 378 to 391 bp for *trn*L-*trn*F IGS. Generally, species of *Utricularia intermedia* aggr. share the same haplotypes for both plastid markers investigated. Three accessions belonging to this aggregate differ for only one substitution or indel nucleotide site, that is, *U. intermedia* RUB4, *U. stygia* ITM1, *U. stygia* ITM2 and *U. stygia* SWA4. Most of the accessions of *U. bremii* share the same haplotypes with one exception: *U. bremii* ITM5, which is close to most of *U. minor* accessions, differing from them only in two or three nucleotide sites. Just one accession of *U. minor*, *U. minor* ITT2, is distant from all the other ones of the same species and its closest haplotypes are those of *U. bremii*, being different in two to five sites. Concerning *U. vulgaris* aggr., all accessions grouped together, but accessions of *U. australis* from Viareggio differ from all the other accessions in five to six sites. With the exception of the two accessions *U. bremii* ITM5 and *U. minor* ITT2, haplotypes of *U. bremii* and *U. minor* are distinct, differing in more than 20 sites (including indels).

ITS sequences ranged from 560 to 614 bp in length. We did not observe double peaks in the electropherograms of ITS sequences of any sample (no polymorphic sites). In the ITS alignment, no constant differences were found between *U. bremii* and *U. minor*. Within *U. intermedia* aggr., *U. intermedia* and *U. stygia* show distinct constant differences in many nucleotide sites (>30). Of the two accessions of *U. ochroleuca*, one shows an ITS profile identical to that of many accessions of *U. stygia*, the other one to that of *U. intermedia*. Concerning *U. vulgaris* aggr., no diagnostic differences were found between accessions of the two species.

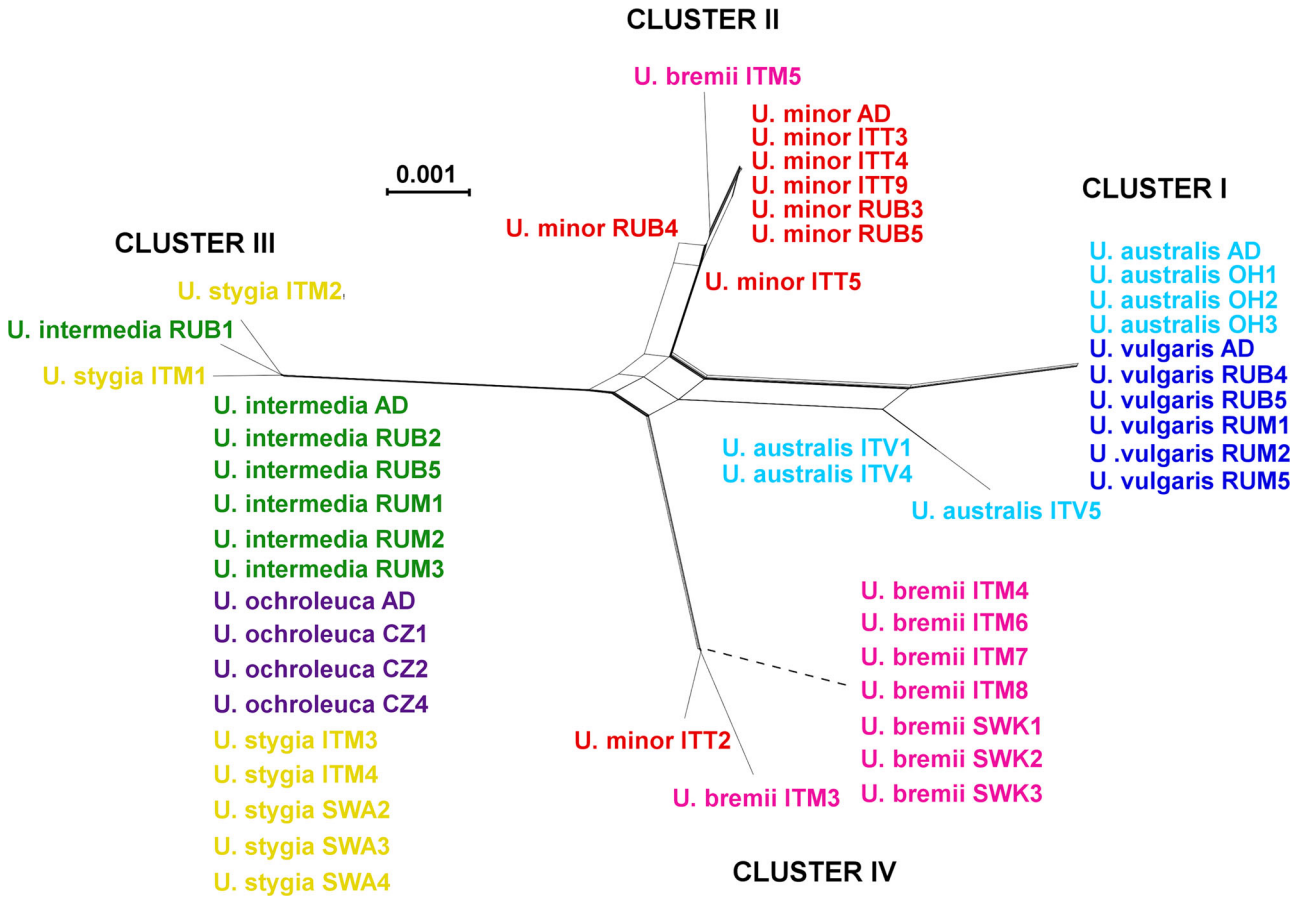

Figure 1. Neighbor-Net plastid network. Network obtained using a combined dataset of *rps*16 intron and *trn*L-*trn*F IGS. Character transformation based on uncorrected *p*-distances method. Edge length are proportional to uncorrected *p*-distance and scale bar represents the number of substitutions per site. Dashed lines link nodes with corresponding multiple sequences otherwise difficult to see graphically. AD = Lubomír Adamec Institute's collection (Třeboň Basin, Czech Republic).

Evolutionary and phylogenetic relationships

In the plastid network, central European species are divided into four well-separated clusters (Figure 1). In cluster I, corresponding to sequences of *Utricularia vulgaris* aggr., the two different species are intermingled, with the exception of *U. australis* from Viareggio, which is separated from all the other sequences. Clusters II and IV are mainly constituted by one species, *U. bremii* and *U. minor*, respectively, with populations mixed to each other, while cluster III includes all species of *U. intermedia* aggr. with most populations sharing the same haplotype.

In the ITS network (Figure 2), three main clusters were found among central European species: cluster I is constituted by species of *U. vulgaris* aggr., cluster II by sequences of *U. intermedia* and one accession of *U. ochroleuca* and finally, cluster III includes the rest of the accessions. This latter cluster is the most diverse, with accessions belonging to *U. intermedia* aggr. and *U. minor* aggr. distributed among more or less different haplotypes. In some cases, accessions of different species from different aggregates can be found within the same haplotype.

The phylogenetic tree (Figure 3) built using cpDNA markers was not able to resolve relationships among the three aggregates. However, it is apparent from cpDNA tree that all clades corresponding to clusters found in the cpDNA network form well-supported clades (PP = 100%), whereas nodes connecting these clades collapsed to a polytomy. Within the clade constituted by *U. australis* + *U. vulgaris* + *U. macrorhiza*, the population from Viareggio, Italy is sister to all other accessions. The latter are further subdivided in two clades: one constituted by *U. macrorhiza*, which is sister to the remaining sequences of *U. australis* and *U. vulgaris*.

In the ITS tree (Figure 4), *U. minor* and *U. intermedia* aggregates form a well-supported clade (PP = 99%) sister to the *U. vulgaris* aggr. clade. *Utricularia minor* aggr. + *U. intermedia* aggr. clade is subdivided in two well-supported clades (100% and 97%, respectively): a clade constituted by *U. intermedia* and one accession of *U. ochroleuca* (*U. ochroleuca* CZ2) sister to *U. bremii* + *U. minor* + *U. stygia* + one accession of *U. ochroleuca* (*U. ochroleuca* CZ1). In the former subclade, *U. intermedia* clusters alone (PP = 96%) and is sister to *U. ochroleuca* (*U. ochroleuca* CZ2); in the latter subclade, relationships among species are mostly unresolved, although a clade constituted by most of the accessions of *U. bremii* and one accession of *U. minor* is found, but with a weak support (PP = 73%). The sisterhood of *U. australis* from Viareggio, Italy to the other accessions within the clade *U. australis* + *U. vulgaris* + *U. macrorhiza* is also confirmed by ITS, despite with a low statistical support (PP = 65%).

Phylogenetic trees built with gaps treated as missing data (not shown) do not provide significant differences as compared to analyses with coded gaps, with two exceptions: a) in the ITS phylogenetic tree, the clade corresponding to *U.*

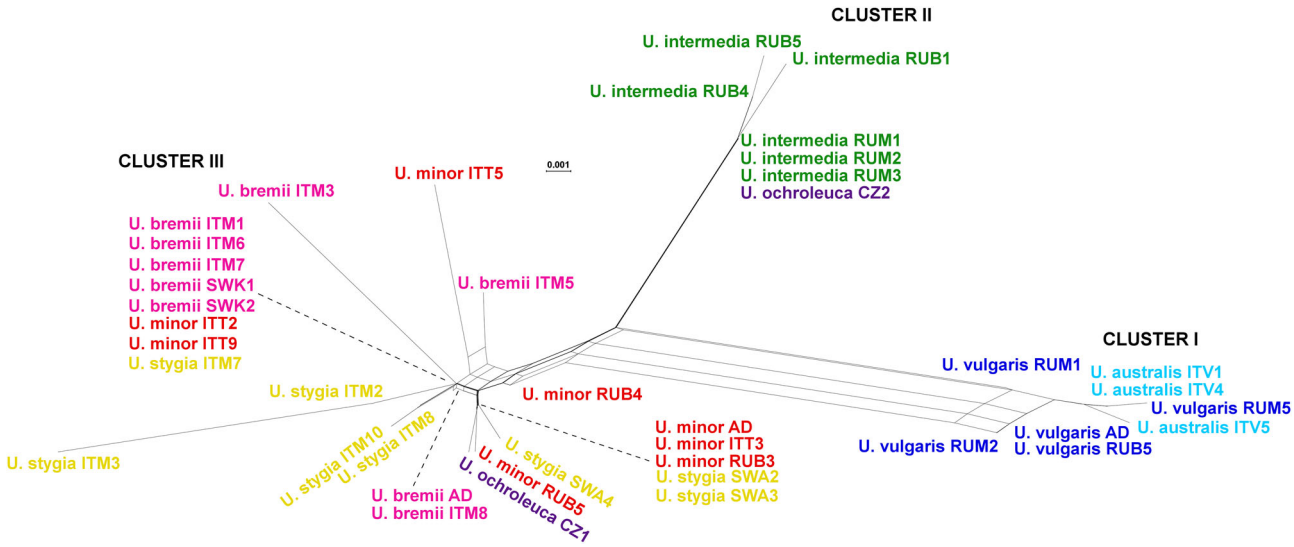

Figure 2. Neighbor-Net ITS network. Character transformation based on uncorrected *p*-distances method. Edge length are proportional to uncorrected *p*-distance and scale bar represents the number of substitutions per site. Dashed lines link nodes with corresponding multiple sequences otherwise difficult to see graphically. AD = Lubomír Adamec Institute's collection (Třeboň Basin, Czech Republic).

vulgaris aggr. is weakly supported (PP = 53%) and b) the weakly supported clade (PP = 73%) containing most of the accessions of *U. bremii* disappears.

Discussion

Concerning barcoding, different haplotypes (for both cpDNA and nuclear rDNA) were found within the same species; conversely, putatively different species shared the same haplotype. For this reason, it was not possible to use such an approach to discriminate all species.

However, by means of the ITS marker, a discrimination between *U. intermedia* and *U. stygia* is possible, although these species are easily recognizable on vegetative morphological grounds as well (Astuti and Peruzzi 2018a). On the contrary, the morphologically overlapping *U. ochroleuca* and *U. stygia* are not distinguished by their ITS sequences. The same sequences of plastid markers are found within *U. intermedia* aggr., making these markers unusable for barcoding.

Within *U. minor* aggr. different cpDNA haplotypes were found, almost perfectly corresponding to the two different species. Indeed, just one out of the nine accessions of *U. bremii* (*U. bremii* ITM5) clustered with *U. minor*, and one out of the nine accessions of *U. minor* (*U. minor* ITT2) clustered with *U. bremii* (Figure 1). The identification of these troubling accessions as well as all other accessions was made based on reliable morphological features, such as the shape of lower flower lip in *U. minor* aggr. Moreover, *U. bremii* ITM5 was collected in Lago Monticolo (Italy), where, after thorough surveys of the site, only *U. bremii* has been reported (Beretta et al. 2011). On the other hand, *U. minor* ITT2 was collected in Passo del Tonale (Italy), where the co-occurrence of *U. bremii* cannot be excluded. Despite this, during our surveys in 2013 and 2014 we only found and sampled specimens showing the flower morphology typical of *U. minor*. Thus, a barcoding approach can be applied for distinguishing *U. bremii* and *U. minor*, considering both *rps*16 and *trn*L-*trn*F markers alignment, but with a little chance of misidentification. Conversely, ITS marker failed to discriminate these two morphologically almost identical (Astuti and Peruzzi 2018a) species. The same occurred in *U. vulgaris* aggr., where neither ITS nor plastid markers were able to provide barcoding regions able to discriminate *U. australis* from *U. vulgaris*, which are morphologically very similar when flowers are missing (Astuti and Peruzzi 2018a). Curiously, both ITS and plastid markers are able to identify the population of *U. australis* sampled in Viareggio (Italy) from other populations.

From an evolutionary point of view, the putative sexual species *U. intermedia*, *U. minor*, and *U. vulgaris* (Taylor 1989) are well separated from each other in both plastid and nuclear networks (Figures 1 and 2), a result that is in accordance with the concatenated tree published by Silva et al. (2018). Regarding the phylogenetic relationships among these species, the ITS tree (Figure 4) shows that species in *U. intermedia* aggr. and *U. minor* aggr. are closer to each other than to species in *U. vulgaris* aggr., again in accordance with the concatenated tree published by Silva et al. (2018). On the other hand, incongruences between plastid and nuclear networks were mostly found (Figures 1 and 2) for the sterile species *U. ochroleuca* and *U. stygia*, supporting the hypothesis of their hybrid origin (Neuman 1900; Thor 1988; Schlosser 2003; Płachno and Adamec 2007). In the studies published by Rahman (2006, 2007), *U. intermedia* and *U. minor* were very closely related, while in our study the former species shows no clear relationship with neither *U. minor* nor *U. bremii*, which are closely related to each other, at least in the ITS phylogenetic graphs.

Although most of the accessions of *U. minor* cluster together, some genetic variation does exist within this species (accession *U. minor* ITT2, see above), as well as in *U. bremii* (accession *U. bremii* ITM5). Most of *U. bremii* sequences cluster separately from all the other sequences in the combined plastid network, while they constitute a separate weakly supported clade (PP = 73%), close to both *U. minor* and *U. stygia*, in the ITS network (Figure 2). The *U. bremii*

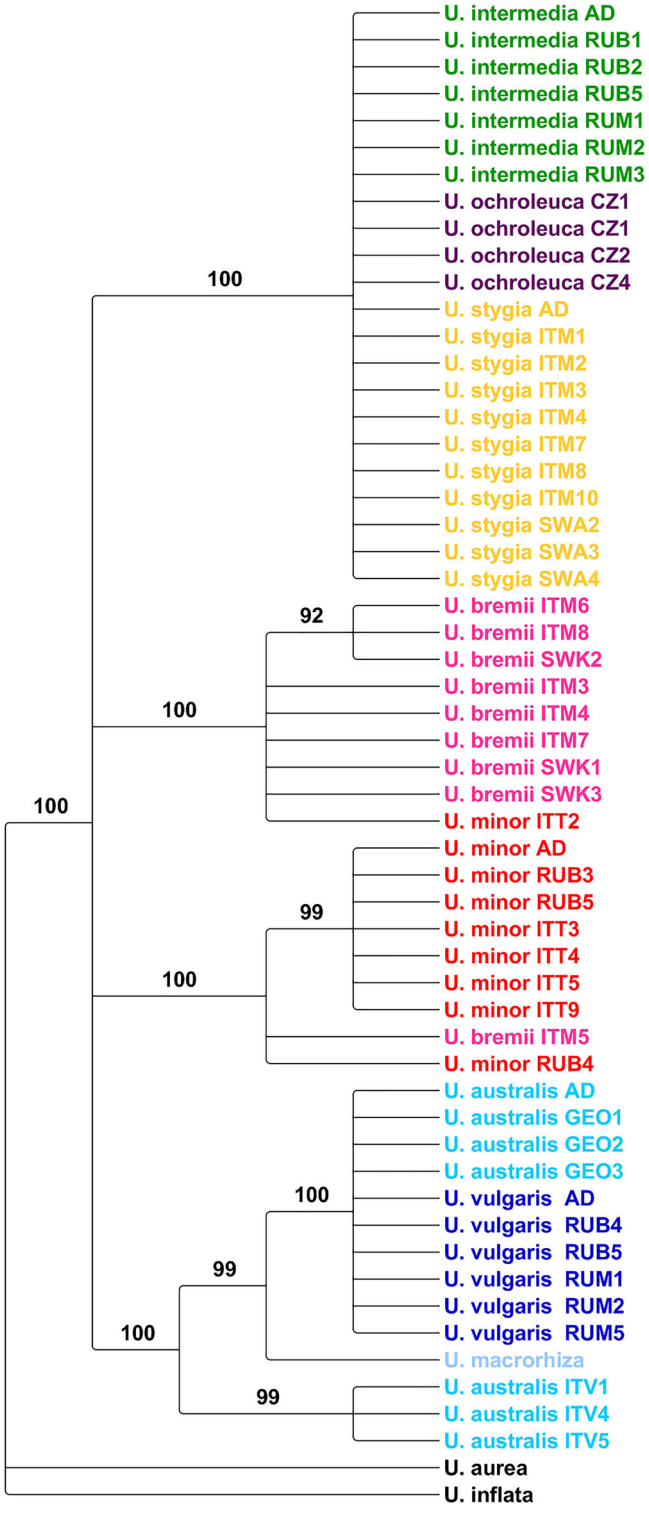

Figure 3. Plastid phylogenetic tree. Bayesian consensus tree inferred from *trn*L-F and *rps*16 plastid markers. Numbers above the branches indicate the posterior probability (PP).

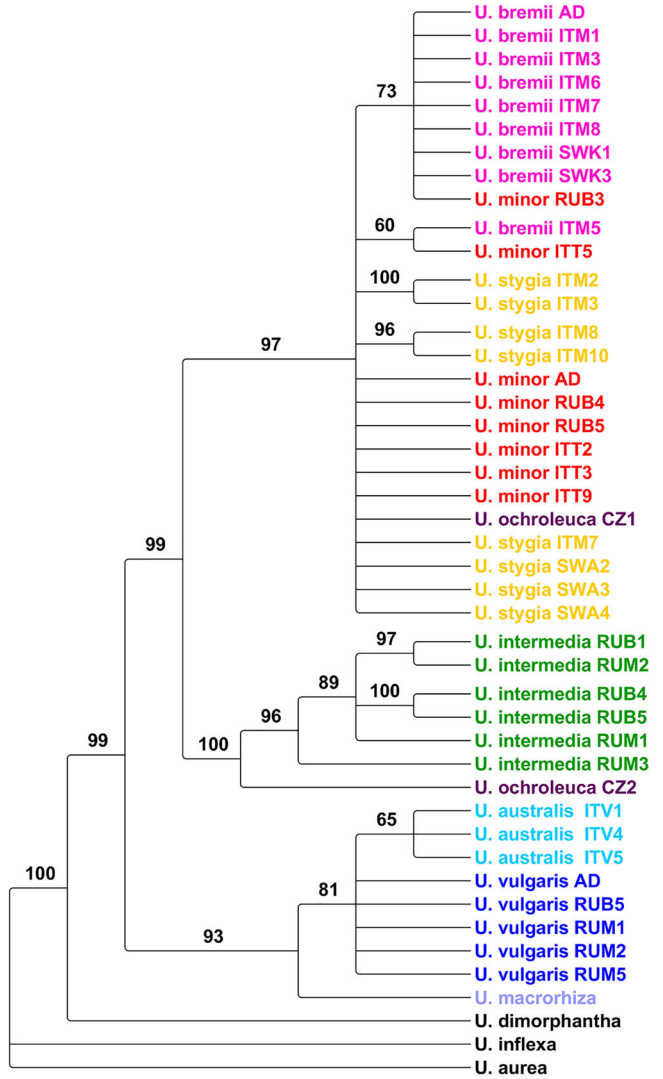

Figure 4. ITS phylogenetic tree. Bayesian consensus tree inferred from ITS rDNA marker. Numbers above the branches indicate the posterior probability (PP).

clade in the phylogenetic tree is mixed with one accession of *U. minor* (*U. minor* RUB3), while a single accession of *U. bremii* (*U. bremii* ITM5) falls outside the *U. bremii* clade, clustering with one accession of *U. minor* with a low support (PP = 60%) (Figure 4). Thus, only the ITS marker is consistent with the high morphological similarity between *U. bremii* and *U. minor*. Possibly, if a hybridization event was involved in the origin of *U. bremii*, seeing *U. minor* as one of the parents, the latter species may have been the male parental species, assuming plastid DNA as maternally inherited in Lentibulariaceae. Nevertheless, also considering morphological results on quadrifid digestive glands reported recently by Astuti and Peruzzi (2018a), *U. bremii* does not show characters intermediate between different species, but rather shows morphological features matching those of *U. minor*. In addition, *U. bremii* and *U. minor* also share the same ITS haplotypes and, sometimes, the same plastid haplotypes, attesting for some gene flow still occurring between these two species (Figure 5). For these reasons, the dysploid apomict hypothesis raised by Taylor (1989) may be reliable in the case of the species pair *U. minor*/*U. bremii*, albeit the circumscription of *U. bremii* as a species different from *U. minor* remains disputable (Astuti and Peruzzi 2018a). Indeed, these species are also generally very similar concerning shoot morphology (Taylor 1989) and share the same flower palate structure (Płachno et al. 2017). Beretta et al. (2014) have recently presented an identification key to central European *Utricularia* using morphology of pollen, which usually bears

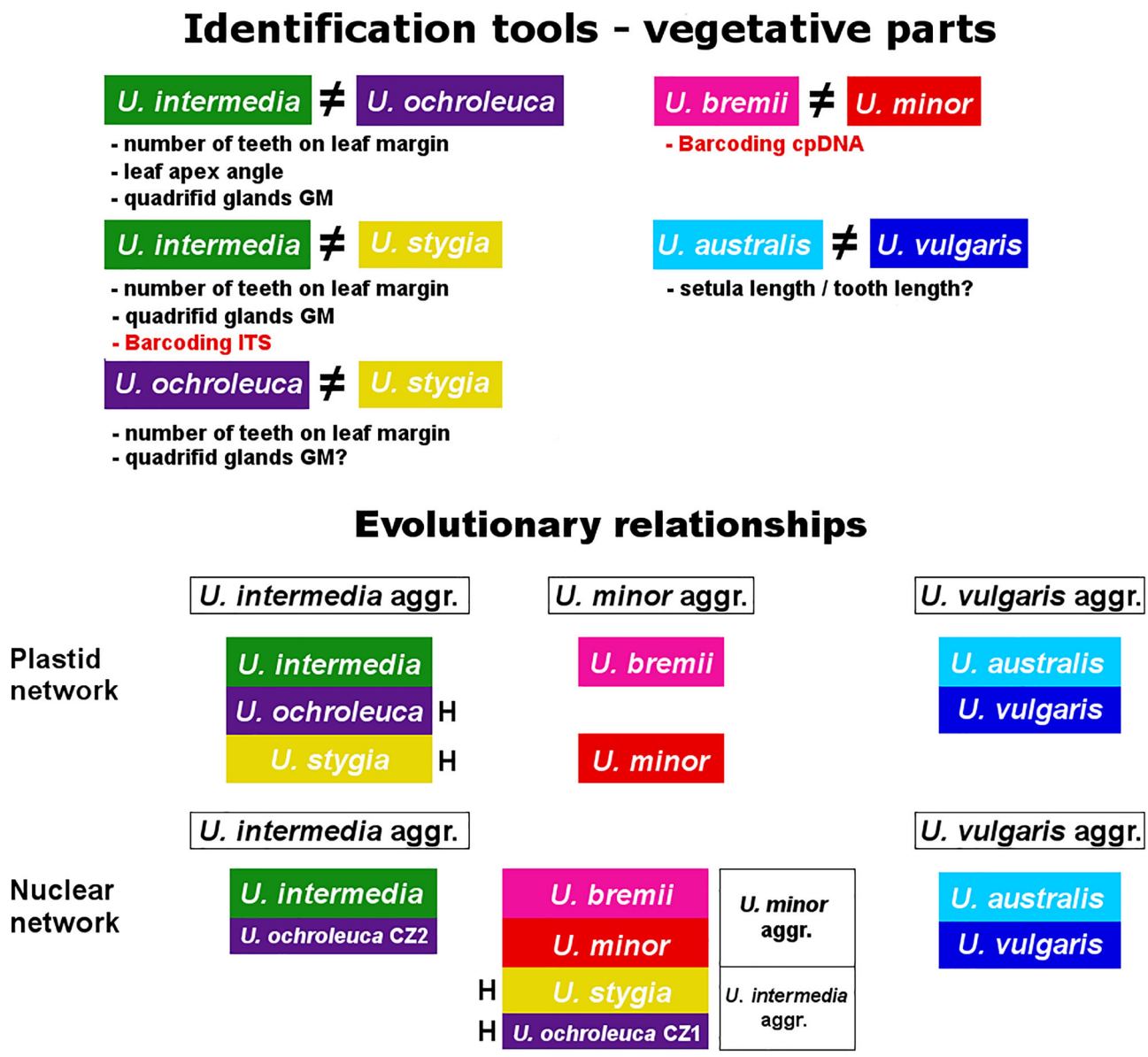

Figure 5. Concluding remarks. Summary of the results found in the study by Astuti and Peruzzi (2018a) and the present study. H = putative hybridogenous taxon according to the present study; GM = geometric morphometrics;? = doubtful discriminating feature.

asymmetric and malformed grains in *U. bremii,* whereas prominently normal and spheroidal grains can be observed in *U. minor*. However, also in this case, flowers are needed for species identification.

Sequences of *U. stygia* cluster with the other species of *U. intermedia* aggr. considering the plastid network (Figure 1) while, in the ITS network, these sequences cluster close to sequences of *U. minor* and *U. bremii* (Figure 2), supporting a putative hybridization *U. intermedia* × *U. minor*, though in *U. intermedia* fruits have been found rarely. An even more puzzling situation was found for *U. ochroleuca*, which is close to species of the *U. intermedia* aggr. in the plastid network (Figure 1), while the only two available sequences cluster distant from each other in the ITS network, where one accession (*U. ochroleuca* CZ1 from Nadějský fishpond, Třeboň Basin, Czech Republic) is close to *U. minor*, the other one (*U. ochroleuca* CZ2 from Ptačí Blato fishpond, Třeboň Basin) to *U. intermedia* (Figure 2). In this case, the incongruences between different networks may be explained by incomplete lineage sorting (Doyle 1992; Maddison 1997; Posada and Crandall 2001; Naciri and Linder 2015). Indeed, ITS may be present in different alleles in *U. ochroleuca* populations and different isolated populations may have divergent copies, paralogues or orthologues, retaining some ancestral copy. For instance, the individual from Ptačí Blato fishpond could have retained a copy of the ITS similar to that of *U. minor* and *U. stygia*, possibly being not much divergent from the ancestral copy present before the separation of *U. intermedia* aggr. from *U. minor* aggr. Conversely, the individual from Nadějský fishpond could have a derivative copy similar to that of *U. intermedia*, originated after the separation of *U. intermedia* aggr. from *U. minor* aggr. Similarly, *U. stygia* could be actually related to *U. intermedia*, despite its sequences are closer to *U. minor* in nuclear network. This could be due to the retention of an ITS copy not much differentiated from the ancestral one present before the separation of

U. intermedia aggr. from *U. minor* aggr. However, in all these cases we should assume that concerted evolution may have been silent, or that it led to the retention of only one copy, but different, according to populations. This latter explanation may be consistent with the massive clonal propagation characterising all these species. However, a possible hybridization origin *U. intermedia* × *U. minor* for plants from Nadějský fishpond ascribed to *U. ochroleuca* cannot be ruled out (Figure 5). Alternatively, both accessions of *U. ochroleuca* may be of hybrid origin *U. intermedia* × *U. minor*, but they are distant in the network because one retained an ITS copy inherited by the male parental species (putatively *U. minor*), while the other retained an ITS copy inherited by the female parental species (putatively *U. intermedia*). Genome size estimations (Veleba et al. 2014) do not support the hybrid hypothesis, since no one of the putatively hybridogenic species (*U. bremii*, *U. ochroleuca*, and *U. stygia*) shows intermediate values between those of the putative parental species (*U. intermedia* and *U. minor*). However, since high mutation rates were found in the genus *Utricularia* (Jobson and Albert 2002; Müller et al. 2004), this kind of data must be taken cautiously.

Sequences of *U. australis* and *U. vulgaris* cluster together (Figures 3 and 4), as expected considering their high morphological similarity (Astuti and Peruzzi 2018a). They also form a well-supported clade together with *U. macrorhiza* in cpDNA tree. However, in this tree, *U. australis* from Viareggio (Italy), is deviating from all other sequences, including the co-specific accessions from Oranienbaum Heide (Germany) and from Třeboň (Czech Republic), which are closer to *U. vulgaris* accessions (Figures 1 and 3). Then, for *U. australis* as well, some intraspecific genetic variation exists, and it is consistent with the hypothesis that each population may represent an apomict unit, not only differing in chromosome number (Taylor 1989), but also in genomic profile. Although not present in Europe, we included *U. macrorhiza* in our analysis because it has been found to be the putative male parental species of *U. australis* (Kameyama et al. 2005). Our analysis confirms the close relationship between *U. australis* and *U. macrorhiza*, but *U. macrorhiza* is closer to *U. vulgaris* in both cpDNA and rDNA trees. As already commented above, this could be explained by the "apomictic hypothesis", or alternatively by incomplete lineage sorting. However, no clear relationships among *U. australis*, *U. macrorhiza*, and *U. vulgaris* can be inferred from our results, as well as from those obtained by Silva et al. (2018). Unfortunately, as there are no sequences of *U. tenuicaulis* Miki in GenBank, we could not include this species in our dataset. Hence we did not have the opportunity to test its role as putative female parental species originating *U. australis*, as suggested by Kameyama et al. (2005).

The contribution of Silva et al. (2018) was fundamental for exploring the potential use of barcoding in such a taxonomically complicated genus. However, barcoding should have a practical and immediate application on environmental administrative problems, for instance for discriminating taxa under protection policies, and it should provide reliable and safe results. Therefore, our case testifies for the need to adopt the barcoding approach with several populations and individuals in highly polymorphic and fast evolving species.

Conclusions

Barcoding has been promoted as a powerful tool for species identification, and its application has been targeted to many fields, including conservation biology. The central European species of *Utricularia* are a striking example of polymorphic taxa affected by identification troubles hampering proper conservation actions. Barcoding with *trn*L-*trn*F and *rps*16 cpDNA appears inapplicable for most of the critical target species, but may be useful for the distinction of *U. bremii* and *U. minor*, even if a small proportion of the haplotypes (barcodes) found in *U. bremii* can match the haplotypes (barcodes) found in *U. minor* and viceversa.

The large intraspecific variability found here for almost all species may be due to a possible hybrid origin of some taxa, or alternatively the mostly sterile European species may be constituted by several morphologically different vegetative apomicts. The hybrid hypothesis is supported by molecular analyses in this study at least for *U. stygia*, and the occurrence of extant hybrids seems to be supported in *U. ochroleuca* as well. Conversely, the apomict hypothesis could explain the differences found between *U. bremii* and *U. minor*. However, some caution is needed when handling molecular results, because of the possible influence of incomplete lineage sorting and other biases affecting the computation of trees and networks. An enlargement of the dataset in terms of both populations and markers (e.g., plastid mat*K* and *rpl*20-*rps*12 IGS) could help to shed light on the hybrid hypothesis and provide more discrimination power.

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References

Astuti G, Peruzzi L. 2018a. Are shoot features of actual diagnostic value in European bladderworts (*Utricularia* L., Lentibulariaceae)? Plant Biosyst. 152(6):1214–1226. https://doi.org/10.1080/11263504.2018.1435573

Astuti G, Peruzzi L. 2018b. Notes on the typification of the names *Utricularia bremii* Heer and *U. intermedia* Dreves & Hayne (Lentibulariaceae). Phytotaxa. 350:172–176.

Beretta M, Rodondi G, Adamec L, Andreis C. 2014. Pollen morphology of European bladderworts (*Utricularia* L., Lentibulariaceae). Rev Paleobot Palinol. 205:22–30.

Beretta M, Simonazzi M, Arrigoni P, Tassara F. 2011. Nuove stazioni di *Utricularia bremii* Heer ex Kölliker (Lentibulariaceae): considerazioni sulla distribuzione e conservazione in Italia. Inf Bot Ital. 43:91–95.

Bergsten J, Bilton D, Fujisawa T, Elliott M, Monaghan M, Balke M, Hendrich L, Geijer J, Herrmann J, Foster GN, et al. 2012. The effect of geographical scale of sampling on DNA barcoding. Syst Biol. 61(5):851–869.

Bilz M, Kell SP, Maxted N, Lansdown RV. 2011. European red list of vascular plants. Luxembourg: Publications Office of the European Union.

Bryant D, Moulton V. 2004. Neighbor-Net: an agglomerative method for the construction of phylogenetic networks. Mol Biol Evol. 21(2):255–265.

CBOL Plant Group. 2009. A DNA barcode for land plants. Proc Natl Acad Sci USA. 106:12794–12797.

China Plant BOL Group 2011. Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. Proc Natl Acad Sci USA. 108:19641–19646.

Doyle JJ. 1992. Gene trees and species trees: molecular systematics as one-character taxonomy. Syst Bot. 17(1):144–163. http://dx.doi.org/10.2307/2419070

Fazekas A, Burgess K, Kesanakurti P, Graham S, Newmaster S, Husband B, Percy DM, Hajibabaei M, Barrett S. 2008. Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. PLoS One. 3(7):e2802.

Fleischmann A. 2012. The new *Utricularia* species described since Peter Taylor's monograph. Carniv Pl Newsl. 41:67–76.

Fleischmann A, Schlauer J. 2014. Die Gattung *Utricularia* in Bayern. Ber Bayer Bot Ges. 84:65–90.

Gariboldi L, Beretta M. 2008. *Utricularia vulgaris* L. e *Utricularia australis* R.Br. due piante carnivore in provincia di Milano. Pianura – Scienze e Storia Dell'ambiente Padano. 23:3–22.

Ghazoul J. 2005. Pollen and seed dispersal among dispersed plants. Biol Rev Camb Philos Soc. 80(3):413–443.

Hebert P, Cywinska A, Ball S, de Waard JR. 2003. Biological identifications through DNA barcodes. Proc Biol Sci. 270(1512):313–321.

Hollingsworth ML, Andra Clark A, Forrest LL, Richardson J, Pennington RT, Long DG, Cowan R, Chase MW, Gaudeul M, Hollingsworth PM. 2009. Selecting barcoding loci for plants: evaluation of seven candidate loci with species-level sampling in three divergent groups of land plants. Mol Ecol Res. 9(2):439–457.

Hollingsworth PM, Graham SW, Little DP. 2011. Choosing and using a plant DNA barcode. PLoS One. 6(5):e19254. https://doi.org/10.1371/journal.pone.0019254

Huson DH. 1998. SplitsTree: a program for analyzing and visualizing evolutionary data. Bioinformatics. 14(1):68–73. Software available from www.splitstree.org

Huson DH, Rupp R, Scornavacca C. 2010. Phylogenetic networks. Cambridge (UK): Cambridge University Press.

Jobson RW, Albert VA. 2002. Molecular rates parallel diversification contrasts between carnivorous plant sister lineages. Cladistics. 18:127–136.

Jobson RW, Playford J, Cameron MK, Albert VA. 2003. Molecular phylogenetics of Lentibulariaceae inferred from plastid *rps*16 Intron and *trn*L-F DNA sequences: implications for character evolution and biogeography. Syst Biol. 28:157–171.

Kameyama Y, Toyama M, Ohara M. 2005. Hybrid origins and F1 dominance in the free-floating, sterile bladderwort, *Utricularia australis* f. *australis* (Lentibulariaceae). Am J Bot. 92(3):469–476.

Kuroiwa T. 1991. The replication, differentiation, and inheritance of plastids with emphasis on the concept of organelle nuclei. Int Rev Cytol. 128:1–62.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al. 2007. Clustal W and Clustal X version 2.0. Bioinformatics. 23(21):2947–2948.

Li DZ, Gao LM, Li HT, Wang H, Ge XJ, Liu JQ. 2011. Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. Proc Natl Acad Sci USA. 108:19641–19646. https://doi.org/10.1073/pnas.1104551108

Lindberg H. 1921. Floristiska meddelanden. Medd Soc Fauna Fl Fenn. 46:103–105.

Linder CR, Rieseberg CH. 2004. Reconstructing patterns of reticulate evolution in plants. Am J Bot . 91(10):1700–1708.

Lodhi MA, Ye GN, Weeden NF, Reisch BI. 1994. A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Ampelopsis*. Plant Mol Biol Rep. 12(1):6–13.

Maddison WP. 1997. Gene trees in species trees. Syst Biol. 46(3):523–536. http://dx.doi.org/10.1093/sysbio/46.3.523

Moeslund B, Løjtnant B, Mathiesen H, Mathiesen L, Pedersen A, Thyssen N, Schou JC. 1990. Danske vandplanter. Myljøstyrelsen, København. Available at https://mst.dk/service/publikationer/publikationsarkiv/1990/jul/danske-vandplanter/

Müller KF. 2005. SeqState – primer design and sequence statistics for phylogenetic DNA data sets. Appl Bioinform. 4(1):65–69.

Müller KF, Borsch T. 2005. Phylogenetics of *Utricularia* (Lentibulariaceae) and molecular evolution of the *trn*K intron in a lineage with high substitutional rates. Plant Syst Evol. 250:39–67.

Müller KF, Borsch T, Legendre L, Porembski S, Theisen I, Barthlott W. 2004. Evolution of carnivory in Lentibulariaceae and the Lamiales. Plant Biol (Stuttg). 6(4):477–490.

Müller KF, Borsch T, Legendre L, Porembski S, Barthlott W. 2006. Recent progress in understanding the evolution of carnivorous Lentibulariaceae (Lamiales). Plant Biol. 8(6):748–757.

Naciri Y, Caetano S, Salamin N. 2012. Plant DNA barcodes and the influence of gene flow. Mol Ecol Resour. 12(4):575–580.

Naciri Y, Linder HP. 2015. Species delimitations and relationships: the dance of the seven veils. Taxon. 64(1):3–16. http://dx.doi.org/10.12705/641.24.

Neuman LM. 1900. *Utricularia intermedia* Hayne × *minor* L. Bot Not. 1900:65–66.

Nieto Feliner G, Rosselló JA. 2007. Better the devil you know? Guidelines for insightful utilization of nrDNA ITS in species-level evolutionary studies in plants. Mol Phylogenet Evol. 44(2):911–919.

Oxelman B, LidéN M, Berglund D. 1997. Chloroplast *rps*16 intron phylogeny of the tribe Sileneae (Caryophyllaceae). Plant Syst Evol. 206(1-4):393–410.

Petit R, Excoffier L. 2009. Gene flow and species delimitation. Trends Ecol Evol (Amst). 24(7):386–393.

Płachno B, Adamec L. 2007. Differentiation of *Utricularia ochroleuca* and *U. stygia* populations in Trebon Basin, Czech Republic, on the basis of quadrifid glands. Carniv Plant Newsl. 36:87–95.

Płachno B, Stpiczyńska M, Krajewski Ł, Świątek P, Adamec L, Miranda V. 2017. Flower palate structure of the aquatic bladderworts *Utricularia bremii* Heer and *U. minor* L. from section *Utricularia* (Lentibulariaceae). Protoplasma. 254(5):2007–2015. https://doi.org/10.1007/s00709-017-1097-9

Posada D, Crandall KA. 2001. Intraspecific gene genealogies: trees grafting into networks. Trends Ecol Evol. 16(1):37–45. http://dx.doi.org/10.1016/S0169-5347(00)02026-7

Rahman MO. 2006. Evaluation of RAPD markers for taxonomic relationships in some aquatic species of *Utricularia* L. (Lentibulariaceae). Bangladesh J Plant Taxon. 13(2):73–82.

Rahman MO. 2007. DNA fingerprinting in *Utricularia* L. section *Utricularia* as revealed by PCR based assay. Int J Bot. 3:56–63.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol. 61(3):539–542. http://dx.doi.org/10.1093/sysbio/sys029.

Rutishauser R, Isler B. 2001. Developmental genetics and morphological evolution of flowering plants, especially bladderworts (*Utricularia*): fuzzy Arberian morphology complements classical morphology. Ann Bot. 88(6):1173–1202.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 4(4):406–425.

Sandionigi A, Galimberti A, Labra M, Ferri E, Panunzi E, De Mattia F, Casiraghi M. 2012. Analytical approaches for DNA barcoding data: how to find a way for plants. Plant Biosyst. 146(4):805–813.

Sang T, Crawford DJ, Stuessy TF. 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. Proc Natl Acad Sci USA. 92(15):6813–6817.

Schlosser E. 2003. *Utricularia stygia* in California, USA, and *U. ochroleuca* at its southern range. Carniv Plant Newsl. 32:113–121.

Silva SR, Gibson R, Adamec L, Dominguez Y, Miranda V. 2018. Molecular phylogeny of bladderworts: a wide approach of *Utricularia* (Lentibulariaceae) species relationships based on six plastidial and nuclear DNA sequences. Mol Phylogenet Evol. 118:244–264.

Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol. 17(5):1105–1109.

Tassara F. 2002. Primo rinvenimento di *Utricularia stygia* Thor (Lentibulariaceae) in Italia e suo confronto con precedenti segnalazioni di *U. ochroleuca* Hartman. Gredleriana. 2:263–270.

Taylor P. 1989. The genus *Utricularia*: a taxonomic monograph. London: Kew Bulletin Series.

Thor G. 1988. The genus *Utricularia* in the Nordic countries, with particular emphasis on *U. stygia* and *U. ochroleuca*. Nord J Bot. 8:219–225.

Tison JM, de Foucault B. 2014. Flora Gallica. Flore de France. Biotope Éditions, Mèze. Available at http://www.biotope-editions.com/index.php?article13/flora-gallica-flore-de-france

Tilney-Bassett RAE. 1978. The inheritance and genetic behavior of plastids. In: Kirk JTO, Tilney-Bassett RAE, editors. The plastids, their chemistry, structure, growth and inheritance. 2nd ed. Amsterdam: Elsevier/North Holland; p. 433–459.

Veleba A, Bureš P, Adamec L, Šmarda P, Lipnerová I, Horová L. 2014. Genome size and genomic GC content evolution in the miniature genome-sized family Lentibulariaceae. New Phytol. 203(1):22–28.

Weising K, Nybom H, Wolff K, Kall G. 2005. DNA fingerprinting in plants: principles, methods and applications. 2nd ed. Boca Raton: CRC Press Taylor & Francis Group.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand D., Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. San Diego: Academic Press.

Zhang A, He L, Crozier R, Muster C, Zhu C. 2010. Estimating sample sizes for DNA barcoding. Mol Phylogenet Evol. 54(3):1035–1039.