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BIOSYSTEMATICS OF EUROPEAN SPECIES OF CARNIVOROUS GENUS *UTRICULARIA* (LAMIALES, ANGIOSPERMS)

DOCTORAL THESIS

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In loving memory of my father Mario

"…it's just a ride…"
Bill Hicks

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ABSTRACT

Utricularia is a genus of carnivorous plants catching its preys using small traps. In Europe, seven aquatic species occur. They rarely flower and the species identification, using only vegetative parts, is difficult. Except flowers, according to literature, teeth on the lateral margin of the ultimate leaf segments should discriminate *U. australis* from *U. vulgaris* ("*U. vulgaris* aggregate"). Concerning *U. intermedia*/*U. ochroleuca*/*U. stygia* complex ("*U. intermedia* aggregate"), the number of teeth on the leaf margin, the ultimate leaf segment apex shape and the quadrifid gland features are considered crucial for species distinction. However, the reliability of these features has never been tested from a quantitative and statistical perspective. About quadrifid glands inside traps, previous authors stated that all the Scandinavian species might be distinguished by their features. The present study was focused on morphological and geometric morphometric investigations in order to quantitatively test the reliability of the features of vegetative parts reported as diagnostic in literature. Also, molecular analysis were performed to the search for DNA barcodes and to reconstruct the phylogenetic relationships, using nuclear ITS and plastidial *trn*L-F IGS and *rps*16 intron markers. Some morphological characters revealed to be potentially useful for species discrimination (e.g. teeth on leaf margin in *U. intermedia* aggregate), while others resulted unreliable. Morphometric analysis of the quadrifid glands was not able to discriminate between the whole set of species, but may be diagnostic for species in *U. intermedia* aggregate. Generally, Barcoding approach failed to discriminate species, even if it could be of some help in *U. minor* aggregate. Indeed, with few exceptions, *U. bremii* shows peculiar DNA regions different from *U. minor* for both plastidial markers investigated. However, interesting hypotheses could be derived from phylogenetic networks and trees obtained, including hybridization events to explain the rise of the mostly sterile species, such as *U. stygia*. This species clusters with the other species of *U. intermedia* aggregate in plastidial phylogenies, while it is closely related to species of *U. minor* aggregate in ITS phylogeny. Also *U. ochroleuca* shows some incongruences considering the different markers, at least for some accessions, pointing to the possible occurrence of hybrids.

INTRODUCTION

The carnivorous plants

Since Darwin's *Insectivorous plants* (1875), carnivorous syndrome in angiosperms has long been investigated, mostly because of its relationship with evolution of exclusive physiological and anatomical traits to cope with peculiar ecological needs (Lloyd 1942, Juniper et al. 1989, Albert et al. 1992, Porembski & Barthlott 2006, Ellison & Gotelli 2009, Król et al. 2012). Indeed, such plants are usually taken as model organisms for studying movement response and glandular secretion (Heslop-Harrison 1975, Dixon et al. 1980, Lüttge 1983, Juniper et al. 1989, Sirová et al. 2003, Vincent et al. 2011b, Poppinga et al. 2013). Usually, carnivory has been linked to the scarcity of nutrients, thus representing an environmental adaptive trait. Indeed, most of the carnivorous plants inhabits environments where nutrients are scarce and light is not limiting, such as lakes, fens, bogs, ponds, etc.

Givnish et al. (1984) proposed the cost-benefit model to interpret the evolution of carnivorous plants. This model deals with the cost of producing trap and digestive structures and the benefit of using an additional source of nutrients, so that carnivorous plants have an energetic advantage over the rest of the plants, if light and water are not limiting. This view was partially questioned by Ellison (2006), who stated that carnivorous plants are at a disadvantage respect to non-carnivorous plants in similar habitats, because they are less efficient in using nutrients. Thus, carnivory appears more as a constrained response to both phylogenetic history and severe ecological conditions.

In angiosperms, carnivory is widespread among different lineages, and probably it originated in at least six different moments of flowering plants evolutionary history (Porembski & Barthlott 2006, Ellison & Gotelli 2009). Among these, also the so-called proto-carnivorous plants, which display features for animal killing without the capability of digesting them (Juniper et al. 1989), have to be included. The strategies adopted for the prey capture are various and patterns of convergent evolution can be generally found considering the phylogenetic relationships between order and families. For example, pitcher-traps are typical of both Sarraceniaceae and Cephalotaceae despite these families are phylogenetically distant. On the other hand, in very closely related genera we can find very different strategies, as in the case of the three genera of Lentibulariaceae. However, it is probable that the different devices used as trap could be all derived from the evolution

of a similar basal structure, i.e. a leaf covered by secretory glands (flypaper sticky model, Ellison & Gotelli 2001).

Carnivorous species are distributed in six orders (families in brackets, classification according to APG III, 2009): Poales (Bromeliaceae and Eriocaulaceae), Caryophyllales (Dioncophyllaceae, Drosophyllaceae, Droseraceae, and Nepenthaceae), Ericales (Roridulaceae and Sarraceniaceae), Oxalidales (Cephalotaceae), Asterales (Stylidiaceae) and Lamiales (Byblidaceae, Martyniaceae, and Lentibulariaceae). Globally, twenty genera include at least one carnivorous or proto-carnivorous species, which are ca. 700 in total (Król et al. 2012).

The family Lentibulariaceae

About half of all carnivorous or proto-carnivorous species are included in Lamiales, almost all belonging to Lentibulariaceae. This family is constituted by only true carnivorous plants, subdivided in three genera, *Pinguicula* L., *Genlisea* A.St-Hil., and *Utricularia* L. The genus *Pinguicula* includes about 85 species widely distributed in the northern hemisphere (Eurasia and Northern America), but with the highest diversity in the wet mountain areas of Central and Southern America (Mexico, Central America, Caribbean and South American Andes) (Casper 1966). These plants catch and digest insects by means of glands, secreting mucilage, located on the upper side of rosette leaves (flypaper traps). *Genlisea* includes 29 species occurring in tropical areas of Brazil and Africa and it feeds on soil protozoa using Y-shaped rhizophylls (modified not-photosynthetic hypogeal leaves), constituted by a vesicular bulb-like basal part and a tubular neck ending in two helically twisted arms (eel-trap or lobster-pot traps, Fleischmann et al. 2010). *Utricularia* is the species-richest genus of the family and the species-richest genus among all the genera including carnivorous species, comprising about 220 species distributed in five out of the six continents of the Earth, lacking only in Antarctica (Taylor 1989, Fleischmann 2012). *Utricularia* feeds on different kinds of freshwater microorganisms, by means of complex goatskin-shaped modified leaves (suction traps, Lloyd 1942).

According to Fischer et al. (2004), Lentibulariaceae are herbaceous plants, terrestrial, epiphytic or aquatic. The roots are present only in *Pinguicula*, with primary roots ephemeral, quickly replaced by adventitious roots. *Utricularia* and *Genlisea* are rootless. Leaves (or leaf-like phylloclads in *Utricularia*, the true leaves are the traps) are rosulate

or scattered on stolons, entire or divided, sometimes heterophyllous. Inflorescences are terminal or lateral, racemose, simple, paracladia sometimes present, often reduced to a single flower, bracteate, prophylls present or absent, sometimes connate. Flowers are zygomorphic. Calyx is 2-4 to 5-partite. Corolla sympetalous, 2-lipped, usually spurred, upper lip entire or 2-lobed, rarely with more lobes, lower lip often with distinct gibbous palate, entire or 2-5-lobed. Stamens 2, in the abaxial half of the flower, alternipetalous, anthers bithecous. Ovary is superior, unilocular, with 2 fused carpels and central placenta. The fruit is usually a capsule. Seeds without endosperm.

Lentibulariaceae are cosmopolitan, with two species of *Pinguicula* holding the northern and southern extremes distribution (*P. vulgaris* L. in Greenland and *P. antarctica* Vahl. in Tierra del Fuego). Generally, the species of this family inhabit nutrient poor habitats like areas with scarce vegetation, stagnant to swiftly flowing waters, epiphytic habitats, including the tanks of bromeliads (Fischer et al. 2004).

All the three genera of the family show a peculiar development as concerns embryo, which is generally reduced and an evolutionary trend of simplification is detectable within the family (Merl 1915). Embryos of *Pinguicula* bear a typical radicle, a hypocotyl, one or two cotyledons and a shoot apex (Degtjareva et al. 2004, 2006), while in *Genlisea* the radicle lacks (Merl 1915) and in *Utricularia* embryos are mass of barely differentiated cells without any lateral organ (Lloyd 1942). Surprisingly, in three species of *Utricularia* sect. *Iperua* ripe seeds bear multiple photosynthetic lateral organs, assuming a characteristic octopus-like shape (Taylor 1989). However, Płachno & Świątek (2010) demonstrated that these structures are not homologous to cotyledons of *Pinguicula*, but to other embryo structures found in *U.* sect. *Utricularia*.

Molecular phylogenetic studies (Müller et al. 2004, Schaferhoff et al. 2010) within Lamiales were not able to identify the immediate sister family of Lentibulariaceae, which resulted included in a weakly supported clade with Acanthaceae, Thomandersiaceae and Martyniaceae, Schlegeliaceae and Bignoniaceae, Pedaliaceae and Verbenaceae. Instead, Lentibulariaceae are clearly monophyletic and are phylogenetically relatively distant from the other families showing carnivorous syndrome, Martyniaceae and Byblidaceae, which also are mutually relatively distant, suggesting that carnivory syndrome has independently evolved more than once in Lamiales (see also Schaferhoff et al. 2010). The monophyly of Lentibulariaceae is supported by the similarity of flower characters and of structures of digestive glands, which are attached to vessels in all genera, contrary to

Byblidaceae and Martyniaceae, where glands rest on at least two epidermal cells (Müller et al. 2004). As concerns relationships within this family, *Pinguicula* is sister to the clade *Utricularia+Genlisea*. Reconstruction of the evolution of morphological characters (Müller et al. 2004) revealed that in all lineages of the family the ancestors were terrestrial, as well as the immediate common ancestor of the family, while submerged and epiphytic conditions are derivative ones. In addition, the putative common ancestor of the family showed basal rosette leaves and a primary root, reduced after germination. Rosette leaf was lost in the adaptation to aquatic environments, as in the most derivative species of *Utricularia*. Similarly, root was completely lost in both *Genlisea* and *Utricularia*, probably because its function was replaced by other structures (leaves, shoots or related modified-organs). Importantly, the more complex trap structure of both *Utricularia* and *Genlisea* originated by folding processes of *Pinguicula*-like leaves. Indeed, as previously postulated (Lloyd 1942, Rutishauser & Sattler 1989), traps of *Genlisea* and *Utricularia* are modified episcidiate leaves and within both genera the abaxially closure of leaves (episcidiation) represents an evolutionary driving process (Müller et al. 2004). This evolutionary trend progressing from an ancestor *Pinguicula*-like to aquatic species of *Utricularia* is confirmed by the ovule anatomy. *Utricularia* has the most specialized ovule, whereas *Genlisea* retains ovule characters, such as free funiculus and endosperm remaining in the ovule, typical of *Pinguicula*, thus inherited from a common ancestor (Płachno & Świątek 2009).

Lentibulariaceae as model organisms in genomic studies

In the last decade, *Utricularia* and *Genlisea* have been thoroughly studied because of their peculiar high rates of molecular evolution (Jobson & Albert 2002, Müller et al. 2004). Particularly for *Utricularia*, it has been hypothesized that these peculiarities are probably linked to the complex mechanism of energy utilization (derived from a unique molecular mutation in a key metabolic pathway) coupled with the so-called relaxed morphology (Jobson et al. 2004, Laakkonen et al. 2006). In fact, a unique nucleotide motif in *cox*I, a subunit of cytochrome *c* oxidase, has been subject to strong selection in *Utricularia* and it could be strictly in connection with the strong energy effort needed for the traps resetting (Jobson et al. 2004). The traps of *Utricularia* are small bladders (1-4 mm in length, usually two-celled thick) filled with water, in which preys are sucked inside once trigger hairs close to trap door are stimulated by any mechanical action (the preys

themselves but even the wind, large metazoans, etc.). These trigger hairs activate the opening of the trap door and, as a result of negative pressure maintained inside the lumen of the trap (Richards 2001, Sirová et al. 2009), the suction occurs. The engulfment of prey within the trap can be extremely fast, around 0.5 milliseconds (Vincent et al. 2011b). It has been demonstrated that traps of *Utricularia* also goes on spontaneous firing without any mechanical stimulation, after a lag period of 5-20 h (Adamec 2011, Vincent et al. 2011a). This spontaneous mechanism could avoid damages to the trap door when magnitude of negative pressure is critical, likely acting as a 'safety valve' (Adamec 2011). After firing, the negative pressure is restored by removing water through the cell walls to restore the compressed shape. This complex mechanism requires a considerable energetic investment, which has been demonstrated by the higher respiratory rates found within the bladders, both with or without prey content, respect to the leaves (Adamec 2006). On the other hand, *Utricularia* is characterized by having a feeble (if any) differentiation between stems, roots and leaves ("relaxed morphology"), often found in other organisms living in aquatic or epiphytic habitats, where the kind of substrate allows itself a structural support, making unnecessary the development of structural tissues (Darwin 1875, Croizat 1960, Ellison & Gotelli 2009). The relaxed morphology and the innovative *cox*I of *Utricularia* probably are the main factors to drive the morphological diversity in this genus.

An alternative hypothesis to explain the high rates of genetic change deals with the highest predictability and frequency of preys in the habitats of *Utricularia* and *Genlisea* respect to the other carnivorous genera coupled with their extreme specialization in prey capture (Müller et al. 2004). According to this hypothesis, *Utricularia* and *Genlisea* take advantage of this large prey availability to uptake a large quantity of amino acids, peptides and nucleotides in his diet. All these molecules represent intact biosynthetic building blocks, being intermediates in various heavily branching pathways. This continuously available external source could lower the selective pressure on the whole machine involved in these metabolic pathways, including the structure of the involved enzymes. In addition, this relaxed selective pressure could have occurred on structural and regulatory genes related to production of roots and leaves, because of the relaxed morphology of these plants.

Similarly, these hypotheses, referred to *Genlisea* and *Utricularia* respect to *Pinguicula*, may apply to other carnivorous plant lineages, where a derivative complex structure of trapping from a simple one (sticky leaf) occurred.

Albert et al. (2010) provided a different explanation for the high substitution rates here discussed and it involved the ROS mutagenic effect. The production of ROS may be due to the same mutation that lead to peculiar *cox*I structure. The unique change of residues (two contiguous cysteines) in *cox*I could modify the functioning of the respiratory chain of mitochondria, decoupling proton pumping and electron transport (Laakkonen et al. 2006). In this way the intramembrane space acts as a reservoir of positive charge, to be spent in ATP once needed, i.e. when the water is actively pumped outside trap lumen for trap resetting. This sequestration of protons could be potentially dangerous, because electrons could leak within mitochondrial lumen and imperfectly react with oxygen resulting in ROS production instead of water (Albert et al. 2010). In support of this hypothesis, a study by Ibarra-Laclette et al. (2011) on *Utricularia gibba* L. demonstrated that high levels of expression of DNA repair and ROS detoxification enzymes coupled with high levels of ROS are produced.

Another peculiarity shown by both *Utricularia* and *Genlisea* is that they include species with the smallest genomes among all angiosperms. *Genlisea tuberosa* Rivadavia, Gonella & A.Fleischm. (1C ≈ 61 Mbp) and *G. aurea* A.St.-Hil. (1C ≈ 64 Mbp) have the record for the smallest genome size in angiosperms (Fleischmann et al. 2014), while in *Utricularia*, *U. gibba* (1C ≈ 81 Mbp) has the lowest value ever found within this genus (Greilhuber et al. 2006). Interestingly, *Genlisea* also includes the species with the largest estimated genome size among all Lentibulariaceae, i.e. *G. hispidula* Stapf with 1C ≈ 1550 Mbp and *G. lobata* Fromm with 1C ≈ 1700 Mbp (Greilhuber et al. 2006, Fleischmann et al. 2014), ca. 24-28 fold largest than the smallest one within the same genus. Veleba et al. (2014) stated that GC content in Lentibulariaceae family covers a substantial part of the entire variation found within the vascular plants (from 30% ca. to 50% ca.; Šmarda & Bureš 2012). For instance, in the only genus *Utricularia* GC content can vary from 34.4 % in *U. purpurea* Walter to 45.1 % in *U. laxa* A.St.-Hil. & Girard. Differently from *Pinguicula*, both *Genlisea* and *Utricularia* show positive correlation between genome size and GC content, suggesting that dynamics of genome shrinkage and magnification could be related to removal or amplification of GC rich non-coding regions. Since non-coding regions are usually GC poor, while coding regions are GC rich, one would expect that species with miniaturized genome have proportionally a high percentage of GC. Instead, in several species of *Genlisea* and *Utricularia* species with small genome size show low GC content, suggesting that, at least for these species, also the coding region

could be involved in the miniaturization process of genomes in Lentibulariaceae (Veleba et al. 2014).

Albert et al. (2010) invoked the same ROS effect called into question for the high nucleotide substitution rates also for this wide genome size span. Indeed, ROS activity can produce breaks on the double stranded DNA structure, resulting in a turnover of non-essential genome space by means of non-homologous recombination. This is consistent with the shorter non-coding sequences and introns and the less repetitive sequences found both for *Genlisea aurea* (Leushkin et al. 2013) and *Utricularia gibba* (Ibarra-Laclette et al. 2013).

Recently, Vu et al. (2015) provided a different explanation of this wide span of the genome size in *Genlisea*, assessing that it was produced by bidirectional evolution of size, starting from a common ancestor of all *Genlisea* species bearing intermediate 1C value (400-800 Mbp) between the extreme values found for this genus. Furthermore, since no significant differences concerning habitats and life strategy related to genome size were found between species with extremely different size, a neutral selection could have occurred in the evolution of genome size in *Genlisea*. In their study, Vu et al. (2015) also found that polyploidization could counteract shrinkage of genome, as a response to loss of essential genes.

The genus *Utricularia*
Distribution and habitats
Since Peter Taylor's monograph on the genus *Utricularia* (Taylor 1989), 21 new species have been described and commonly accepted until now (Fleischmann 2012, Jobson 2012, 2013, Delprete 2014). Thus, the total number of species of *Utricularia* is 235 ca. The genus occurs in almost every country in the world, but it is generally absent in arid regions and oceanic islands. The latitudinal extremes are represented by Arctic Circle to the north and by Stewart Island (New Zealand) to the south at ca. 47°30' S. The vast majority of the species is found in tropical and subtropical regions, with the largest diversity, as well as the largest number of species, in South America (Brazil and the Guianas) (Taylor 1989); for instance, only in the small island of Trinidad 19 species occur. According to Guisande et al. (2007), biogeographic neighbouring areas share a few number of species and the number of species shared by non-neighbouring areas is extremely low, indicating that many of them are endemic. Only two species, *U. subulata* L. and *U. gibba* L. are

distributed in many biogeographic regions: the former is probably the most widespread species, lacking only in Palearctic regions (it is reported in Portugal, where it is probably introduced), whereas the latter is present in all biogeographic regions (Taylor 1989). The exception to this high number of endemism is represented by the western Palearctic Region, where only one species is endemic, i.e. *U. bremii*. It is still unknown where is the centre of origin of this cosmopolitan genus, but phylogenetic studies indicate that it was probably Neotropical, with further dispersion in Afrotropical and then in Australasian regions and, subsequently, in the rest of the regions (Jobson et al. 2003, Müller & Borsch 2005).

Bladders need water for working, so that all the species of *Utricularia* are more or less linked to water for surviving. However, this does not necessary means that they are all aquatic plants, as a matter of fact most of the species (more than 50%) are considered terrestrial (Guisande et al. 2007). According to Taylor (1989), six different categories of species can be recognized according to their habitats: terrestrial, epiphytic, lithophytic, rheophytic, affixed aquatic and free aquatic. As already warned by Taylor himself, it is rather difficult to categorize species in strictly different habits and habitats. Indeed, several species can occur in different habitats and for many species information about habit and habitats is still vague. For example, *U. minor* either can live as free aquatic or affixed aquatic, and *U. ochroleuca* can be either free aquatic or terrestrial. However, these categories give an overall idea of species ecological adaptation and, in many cases, fit very well with species characteristics. Terrestrial species grow in wet soils seasonally inundated because their closeness to waterbodies. However, they can experience long periods of soil drought. Epiphytic species live on other plants, including the pool of waters in the leaf axil of bromeliads and other plants. Litophytic species live on outcrop rocks. Both litophytic and epiphytic plants usually need permanent or seasonally high humidity conditions. Rheophytic species occupy a peculiar niche on swiftly flowering waters and they are equipped with organs anchoring plants to the substrate (usually rock). Then, these plants can be somehow considered partly as aquatic and partly as litophytic. Affixed aquatic species have all or most of their traps borne on shoots, which are anchored to the soil or another more or less solid substrate. Free aquatic species are constituted by free-floating plants, without any part of the plant in contact with the ground, thus being the only true aquatic species. As already said, terrestrial is the most species-rich category, including more than a half of species, followed by free aquatic, which includes ca. 15%

of the species. The category with the lowest number of species is the rheophytic one, including only three species, one of which is usually terrestrial and another one can be found on wet soils. Guisande et al. (2007) analysed morphological characters in the different habitat contexts using Discriminant Analysis and they found that only species within epiphytic (bearing large leaves) and species belonging to free aquatic category (large stolons) can be discriminated, while the rest of the species massively overlap. In almost all groups, high overlap with terrestrial group was recovered, indicating that many species have the capability to be terrestrial and to live in another habitat. Terrestrial habit is considered the plesiomorphic one, while aquatic is apomorphic. The proportion of terrestrial species is neatly higher in Neotropical region, supporting the hypothesis that here is located the centre of origin of the genus. On the other hand, aquatic species are proportionally higher in northern hemisphere, consistently with the putatively derived condition.

As concerns environmental characteristics of the habitats, data in literature (see Guisande et al. 2007 and literature cited therein) are available almost only for aquatic plants and they were consistent with the classical view that carnivorous plants live in environment with low concentrations of nutrients.

Systematics and evolution

Exclusively relying on morphological features, Taylor (1989) recognized 35 sections within the genus *Utricularia*, subdivided in two main subgenera: *Polypompholyx* (Lehm.) P.Taylor and *Utricularia*, the former characterized by species with 4-lobed calyx instead of 2-lobed as in the rest of the species. A molecular phylogenetic study by Müller & Borsch (2005) on *trn*K intron, a plastidial marker, provided evidence for recognition of the subgenus *Bivalvaria* Kurz besides the two ones proposed by Taylor. If this further subgenus had not recognized, the subgenus *Utricularia* would be paraphyletic. In addition, subgenus *Polypompholyx* should include also section *Pleiochasia* Kamiénski (formerly included in subgenus *Utricularia* by Taylor) to circumscribe a monophyletic group. This was also supported by Jobson et al. (2003) using other plastidial markers (i.e. *trn*L-*trn*F intergenic spacer and *rps*16 intron). Nevertheless, molecular phylogenetic studies mostly confirmed the circumscription of sections made by Taylor, with some exceptions (Jobson et al. 2003, Müller & Borsch 2005). Section *Iperua* P.Taylor resulted polyphyletic, while if merged with species of section *Orchidioides* A.DC., this would be

a well-supported clade. Such a treatment of section *Orchidioides* including also *Iperua* was already proposed by Kamiénski (1895). Similarly, section *Foliosa* Kamiénski should be expanded to include also section *Psyllosperma* P.Taylor, and section *Vesiculina* (Raf.) P.Taylor should be expanded to include also section *Setiscapella* (Barnhart) P.Taylor. Other incongruences between molecular studies and Taylor's section delimitation exists, but these incongruences are also present between the different trees calculated using different markers, not clearly allowing any further change to Taylor's classification.

According to Müller et al. (2006), both morphological and molecular studies agreed in considering terrestrial life form as ancestral, while epiphytes and litophytes are derived and evolved independently in section *Orchidioides* and *Phyllaria* (Kurz) Kamiénski. Also the aquatic forms are derived and free-floating ones seem independently evolved within sections *Vesiculina* and *Utricularia*. In addition, in both these sections a parallel trend led to aquatic forms by transgression from terrestrial to affixed aquatic and finally to suspended species. A parallel trend involving evolution of rheophytes from terrestrial forms probably took place independently in sections *Avesicarioides* Komiya and *Avesicaria* Kamiénski. Regarding the putative geographic origin of the genus, current data suggest a south American origin, in line with the hypothesis of a neotropical origin of the sister genus *Genlisea* (Jobson et al. 2003, Müller & Borsch 2005). Generally, phylogeny fits with morphological variability of features of vegetative parts within the genus (Jobson & Albert 2002, Jobson et al. 2003). The sections *Polypompholyx* and *Pleiochasia* appear to be plesiomorphic according to both morphological and molecular studies (Müller & Borsch 2005). Indeed, species of these sections show a putative ancestral rosulate habit of leaves, opposite to the more complex shoot-leaf system. Such a leaf-shoot system reaches its most derivative structure in species of section *Utricularia*, which are characterized by exclusive stoloniferous habit of vegetative parts. In this genus, section *Utricularia* appears to be the end-point of the evolutionary line also considering the pollen architecture (Taylor 1989). Unfortunately, the placement of the rest of the species between the extremes represented by section *Polypompholyx* and *Utricularia* remains unclear (Taylor 1989, Müller & Borsch 2005).

Karyological aspects in *Utricularia* are poorly known and karyotypes are almost unknown. First chromosome numbers in *Utricularia* were counted by Reese (1952) in *U. australis* R.Br., *U. minor* L. and *U. vulgaris* L., all reported with $2n = 36\text{-}40$. Since then, only 13% of the members of the genus have been counted (Tanaka & Uchiyama 1988,

Casper & Manitz 1975, Rahman et al. 2001, Veleba et al. 2014, Rodrigues da Silva et al. 2015). This low number of counts is mainly due to very small size of chromosomes, to the lack of roots (so that rhizoid apex and shoot apex are used for somatic chromosome counts) and to difficulties of staining chromosome with standard dyes, such as orceine, carmine and Giemsa (Kondo 1971). To date, only 32 species of *Utricularia* have at least one chromosome count, with chromosome numbers ranging from $2n = 12$ in *U. scandens* Benj. (Subramanyam & Kamble 1968) to $2n = 80$ in *U. aurea* Laur. (Tanaka & Uchiyama 1988). *Utricularia* sect. *Utricularia*, with 14 species having at least one chromosome count in literature, is the most represented section. As already reported, the genus *Utricularia* shows extremely small genomes for almost all the species estimated up to now, which are 72 (Greilhuber et al. 2006, Veleba et al. 2014). 1C values range from 79 Mbp in *U. purpurea* to 706 Mbp in *U. caerulea* L., so that all the species have a genome large less than 1000 Mbp.

General morphology

As shown by Rutishauser & Isler (2001), *Utricularia* represents a striking example of organs heterotopy in plants. The difficulty to clearly delimit leaves and stem in these plants is paradigmatic of the so-called "relaxed morphology" or "relaxed body plan". For this reason, it is convenient to establish a consistent terminology of the various organs, as suggested by Taylor (1989).

Despite the rootless condition characterizing all species, most of them show organs similar in aspect and functioning to roots, termed commonly *rhizoids*. Their function is usually to anchor the rest of the plant body to the substrate and they are usually found at the basis of the inflorescence peduncle. They are absent in the most primitive species with rosulate leaves, while they are strong in rheophytic species and provided of adhesive trichomes. Strangely, they are also present in some free aquatic species in section *Utricularia*, but their function is unknown and may be interpreted as a vestigial structure (Raynal-Roques & Jérémie 2005).

A proper vertical stem in *Utricularia* is found only in the putative primitive species belonging to section *Polypompholyx*, thus always related to rosulate leaves habit. However, with very few exceptions (represented by primitive species in subgenus *Polypompholyx*) all the species bear stolons, which represent the most prominent part of the plant. They are different according to habitat and phylogeny, ranging from few

millimetres to several meters in length (in free aquatic species) and they are borne underground or they float on the water surface, largely branching and forming dense mats. From stolons usually leaves and inflorescences arise, as well as other specialized organs such as air shoots, tubers, rhizoids and sometimes traps. Some species bear different kind of stolons on the same plant, often with different functions. A clear example of this are the dimorphic stolons in affixed aquatic species of section *Utricularia* such as *U. minor* L. and *U. intermedia* Hayne. These species bear green photosynthetic stolons, with few or no traps, floating on the water surface or laying in wet soils and pale not photosynthetic stolons, with numerous traps and buried in the substrate.

Taylor (1989) and previous authors (Goebel 1891, Lloyd 1942) defined as *leaves* those leaf-like structures arising from near the base of the peduncle. They are of various forms according to habit and phylogeny. The vast majority of leaves are small (few millimetres to few centimetres), petiolate, with linear to obovate lamina, but also shapes such as reniform, peltate and also leaves dichotomously, pinnately or palmately divided in more or less laciniate segments occur. Laciniate divided leaves are typical of aquatic species of section *Utricularia*, which are the remarkable examples of heterotopic character of the body plan, where a clear distinction between stolons and leaves is hard to define. In some species, leaves can be dimorphic and can assume different functions (e.g. *U. hispida* Lam. and *U. mirabilis* P.Taylor). Leaves can be solitary, grouped in rosettes arising from the nodes of the stolons or randomly scattered along the stolons.

An organ exclusive of species of section *Utricularia*, living at temperate climate, is the *turion*. The turion is somewhat a miniaturized plant constituted by a very short axis densely equipped with modified leaves. Similarly to other aquatic species (e.g. *Aldrovanda vesiculosa* L. and *Myriophyllum* L. sp. pl.), turions are produced at the apex of the stolon as organ of persistence to cope with low winter temperature. They are of different shape, but usually globoid, and different size, ranging from less than a millimetre to 2-3 centimetres, according to species. They are extremely important for those species that are mostly sterile and characterized by massive clonal reproduction, favouring the dispersal of these plants (e.g. *U. australis*).

Traps are the most studied structures in *Utricularia* (Withycombe 1924, Lloyd 1942, Sydenham & Findlay 1973, Taylor 1989, Le Strat-Broussaud 2000, Sirová et al. 2003, Reifenrath et al. 2006, Vincent et al. 2011a,b, Poppinga et al. 2013), because of their importance for life strategy, but also for taxonomical reasons. They occur on all species

and, generally, they work in the same way in all of them (Lloyd 1942, Heslop-Harrison 1976). Their taxonomical value is often related to their position on the organs of vegetative part. They can arise from the stem or from the base of the peduncle, from the leaves or from the stolons, at nodes or at internodes, and sometimes even from the rhizoids (Taylor 1989). Traps are globose to ovoid shaped, varying in size from 0.2 mm ca. to 1.2 cm in length, usually with stalks, which show a large variability in length, rarely sessile. They have a mouth, which corresponds to the point of entrance of the prey, located respect to the stalk in various position according to species (basal, terminal or lateral). Position of mouth can also vary within a single species, in accordance with shape, in those with polymorphic traps. The large variability seen for shape, stalks, and mouth position, it is found also in appendages. Appendages occur just outside the mouth, in the oral area of the trap, extending as a tentacle. The appendages along with internal glands represent the most important character for Taylor taxonomic treatments of the whole genus (1989). In the early branching section *Pleiochasia* (subgenus *Polypompholyx*), appendages are largely variable between species, with polymorphism occurring also within a single species. On the other hand, in the most derivative section *Utricularia*, despite the general large morphological variability between species, there is a strong similarity between appendages of all species. However, in this section the whole trap feature is rather homogeneous. They occur laterally in finely branched shoots, with mouth in lateral position and rigid branched appendages, also called *antennae*, on the dorsal side of the mouth (Taylor 1989, Reifenrath et al. 2006); maybe they guide prey towards the entrance of the trap (Darwin 1875, Lützelburg 1910). As concerns the remaining species, each section generally has its own peculiar organization of appendages, strongly affecting Taylor's circumscription.

Glands within the traps are known since Darwin's time (1875) and their function has been discussed by many authors (Thurston & Seabury 1975, Fineran 1985, Le Strat-Broussaud 2000, Sirová et al. 2003, Vintéjoux & Shoar-Ghafari 2005, Płachno et al. 2006). They are trichomes and usually of two kinds, the bifid glands and the quadrifid glands, both occurring on the inner surface of the bladder and constituted by a basal cell surmounted by, respectively two and four, narrow and long cells (*arms*). Bifid glands, located on the threshold region (just inside the door), have a role in water removing for trap resetting, probably acting like salt-excreting cells favouring water removal through osmosis (Sasago & Sibaoka 1985a,b). Instead, quadrifid glands have a direct role in carnivory,

secreting digestive enzymes and adsorbing nutrients from prey. Numerous enzymes have been found to be secreted by these glands, maybe the most important is phosphatase, which shows the highest and predominant activity (Sirová et al. 2003, Płachno et al. 2006, Adamec et al. 2010). According to Taylor (1989), generally, quadrifid glands can assume different shapes, mostly depending on orientation of long arms. Some exceptions to 2-armed and 4-armed organization is provided by species in section *Stomoisia* (Raf.) Kuntze, where on the threshold glands are 1-armed glands and 2-armed in the rest of the bladder, and in section *Stylotheca* A.DC., where all the internal glands are 1-armed. Furthermore, in some species there is a transition from 1-armed glands on the threshold to 2-armed, 3-armed and 4-armed glands elsewhere within the trap. However, gland pattern seems to be quite constant in most of the sections. Nevertheless, in section *Utricularia*, different patterns can be found according to species, and at least for northern European species, the relative disposition of the arms has been considered of high taxonomic value (Thor 1988).

Utricularia prey spectra

In *Utricularia*, it might be argued that captured organisms are preys which provide nutrients (N and P), scarcely available in the habitats where these plants are living, a typical strategy for carnivorous plant species (Darwin 1875, Sorensen & Jackson 1968). Species of *Utricularia* have been reported to capture different kinds of prey, both zooplanktonic and phytoplanktonic organisms (Hegner 1926, Schumacher 1960, Sorensen & Jackson 1968 Andrikovics et al. 1988, Harms 1999, Mette et al. 2000, Peroutka et al. 2008, Alkhalaf et al. 2009). Differences between terrestrial and aquatic species were found, with the former feeding mostly on rotifers and protozoans and the latter showing a wider range of prey spectra (Seine et al. 2002). Probably, metazoans inside the traps (mostly crustacean copepods and cladocerans, rotifers, dipter larvae, etc.) or ciliates, when plants were experimentally grown in poor nutrient conditions (Sorensen & Jackson 1968), might be seen as a source of nutrient. However, according to recent studies, this interpretation is feeble to account for the complex and diverse microorganism communities (green algae, cyanobacters and other bacteria, euglenas, diatoms, ciliates and other protozoans) that inhabit the bladders of aquatic species. In addition, several studies suggested limited importance of preys as a source of nutrients for plant growth in aquatic species (Sorenson & Jackson 1968, Kosiba 1992, Englund & Harms 2003,

Adamec 2008, Adamec et al. 2010). Speculations about the influence of these microorganisms on the plant physiology have been made, not just seeing them as simple preys, but also as mutualists, commensals and even parasites (Hegner 1926, Mette et al. 2000, Peroutka et al. 2008, Alkhalaf et al. 2009, Sirová et al. 2009, 2010, 2011, Płachno et al. 2012). Oxygen concentration near zero in traps of aquatic bladderworts (Adamec 2007a) probably determines the type of organisms that can live inside. First, those that cannot tolerate such oxygen concentration levels, as crustacean and ostracods, will die, and are thus potential prey. Other organisms living in *Utricularia* traps (bacteria, algae, protozoa, rotifers) are on the contrary more tolerant to conditions of long-lasting anoxia interrupted by limited periods of higher oxygen concentration (Adamec 2007a). In species of *Utricularia* living in nutrient-poor water, algae can represent the major food, reaching up to 80% of the entire diet (Peroutka et al. 2008). The complex microbial communities found within traps of many aquatic species of *Utricularia* can also provide an amount of hydrolytic enzymes (e.g. phosphatase), contributing to the digestion of prey (Richards 2001, Sirová et al. 2003, 2009, 2010, 2011, Caravieri et al. 2014). In order to feed these commensal microorganisms, aquatic bladderworts can supply great amount of organic compounds (carbon exudates) to traps (Sirová et al. 2009, 2010, 2011). A particular role in the food web within the traps can be assumed by ciliates, which feed on detritus and control the abundance and biomass of bacteria. Indeed, the number of ciliates, as well as bacteria (prey for ciliates), seems to increase with the trap age. In many cases, ciliates are not digested by the plant and they even reproduce inside the traps (Alkhalaf et al. 2009, Sirová et al. 2009, 2010, Płachno et al. 2012).

European species of *Utricularia*

In Europe only seven native species of *Utricularia* occur: *U. australis* R.Br., *U. bremii* Heer, *U. intermedia* Hayne, *U. minor* L., *U. ochroleuca* R.Hartm., *U. stygia* Thor, and *U. vulgaris* L. *Utricularia gibba* L. and *U. subulata* L., recorded for Iberian Peninsula, are not included in this list, since their autochthony is doubtful and they have been reported as introduced and naturalized (Taylor 1989, Paiva 2001). European species are all aquatic, inhabiting environments often threatened by human activities. At European level, all these species are included in the Red Lists, as *Data Deficient* (*U. bremii, U. ochroleuca, U. intermedia*, and *U. stygia*) or as *Least Concern* (*U. australis, U. minor*, and *U. vulgaris*) (Bilz et al. 2011).

Considering systematic aspects, these species are mainly distinguished by the flowers (Fig. 1A), particularly by the shape of lower lip and the spur (Thor 1988, Taylor 1989, Tassara 2002, Schlosser 2003, Płachno & Adamec 2007). As concerns the shoots (Fig. 1B), the vegetative parts of the plants, we can subdivide the whole set of European species in 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*. The species in *U. intermedia* aggr. show dimorphic shoots (Adamec 2007b): pale carnivorous stolons, usually buried in the substrate or intermingled with plant material, with numerous traps and very few (0-2), reduced leaves; green or reddish photosynthetic stolons lying above the substrate, bearing few or no traps and with palmato-dichotomous leaves with teeth along the margin. The species in *U. minor* aggr. are characterized, as species in *U. intermedia* aggr., by slightly and occasionally dimorphic stolons, green and pale, respectively living above and beneath the substrate; the pale ones with numerous traps and reduced leaves, the green ones with numerous traps and palmato-dichotomous leaves without teeth on the margin. The *U. vulgaris* aggr. includes free-floating species with only monomorphic green stolons, bearing numerous traps on leaves divided at the base in two unequal primary segments, each ± pinnately divided, the secondary segments dichotomously divided into further segments.

For several species (see below), it has been proposed a hybrid origin as well several hybrids have been suggested to occur among European species (Neuman 1900, Lindberg 1921, Schlosser 2003, Płachno & Adamec 2007). Taylor (1989) rejected this view and assessed that the so-called 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).
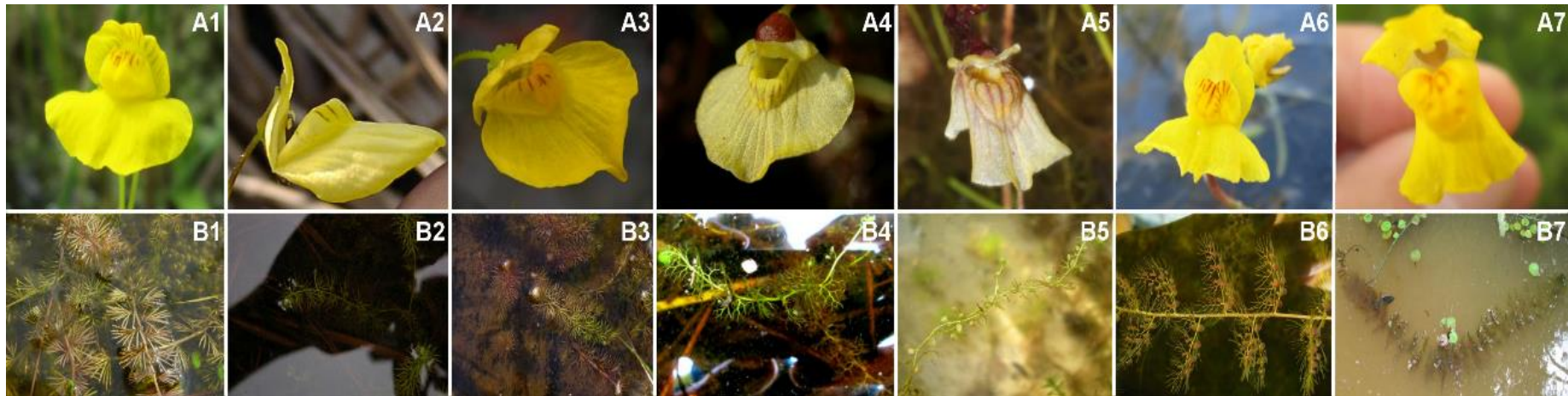
Figure 1. European species of *Utricularia*. Flowers of A1) *U. intermedia* (photo by G. Astuti), A2) *U. ochroleuca* (photo by J. Schlauer), A3) *U. stygia* (photo by A. Fleischmann), A4) *U. bremii* (photo by P. Arrigoni), A5) *U. minor* (photo by P. Arrigoni), A6) *U. australis* (photo by G. Astuti) and A7) *U. vulgaris* (photo by G. Astuti). Stolons of B1) *U. intermedia* (photo by A. Fleischmann), B2) *U. ochroleuca* (photo by A. Fleischmann), B3) *U. stygia* (photo by A. Fleischmann), B4) *U. bremii* (photo by G. Astuti), B5) *U. minor* (photo by A. Moro) B6) *U. australis* (photo by G. Astuti) and B7) *U. vulgaris* (photo by G. Astuti).

Species description

The description of the species presented below is based on Taylor (1989), except where indicated. The description of *U. stygia* is based on Thor (1988), except where indicated. See Appendix I for species synonyms.

Utricularia intermedia **aggr.**

Utricularia intermedia **Hayne** in Schrader, Journal für die Botanik 3: 18 (1800).

Protologue: *Utricularia nectario conico, labio superiore integro palato duplo longiore, foliis tripartitis, laciniis capillaribus dichotomis.*

Utriculari vulgaris minor. Ehrh. herb. n. 91. Habitat in inundates prope Berolinum et Upsaliam. Floret Iunio.

Caulis teres, dichotomus, sub aqua repens, e propagine ovata paulo curvata e squamis tripartitis constructa prodiens et radiculas solitaris filiformis emittens. Ampullae subrotondo-oblongae, compressiuscule radiculis vel cauli defoliato, nunquam vero foliis, affixae. Folia tripartita: laciniae capillares, dichotomae, margine undique setis solitariis minutissimis obsitae. Pedunculus scapiformis, erectus teres bi- vel triflorus et supra medium squama cordato-subrotunda praeditus. Bractea sub quouis pedicello cordato-subrotunda, concava. Cal. Perianthium diphyllum persistens, foliolis ovatis acutis concavis. Corolla monopetala, personata sulphurea: labium superius ovatum, integrum, obtusum, erectum, striis purpureis pictum; labium inferius subrotundum, planiusculum, deflexum; palatum subrotundum, striis purpureis notatum. Nect. Calcar e corolla basi productum, conicum, labio inferiori adpressum. Stam. Filamenta duo, incurvata. Antheraee liberae, uniloculares. Pist. Germen subrotundo-ovatum. Stylus cylindraceus brevis. Stigma ut in precedente. Pericarpium et semina precedent simillima, sed paulo minora.

Lectotype (designated by Taylor 1989: 605): Germany, Berlin, Ehrh. herb. no. 91 (not located). Note: in accordance with Art. 9.23 of the International Code of Nomenclature for algae, fungi and plants, ICN hereafter (McNeill et al. 2012), only for lectotypifications after 1 January 1990 the specification of the herbarium where the type is conserved is needed. In his monograph, Taylor (1989) designated as type the Ehrhart collection already indicated in the protologue by Hayne. Hence, this can be considered as a first-step lectotypification (Art. 9.17, Ex. 12 of the ICN, McNeill et al. 2012). I found a sheet at the

herbarium of Komarov Institute in St. Petersburg (LE! see Fig. 2) hosting a specimen whose label corresponds to the collection indicated in the protologue and designated by Taylor. This could be designated as lectotype in a second-step lectotypification (Art. 9.17, Ex. 12 of the ICN, McNeill et al. 2012).

Small perennial, usually affixed aquatic. Rhizoid usually present, few, filiform, a few cm long, 0.2-0.7 mm thick, bearing numerous short, dichotomously divided branches, the ultimate segments minutely papillose, shortly cylindrical, with apex obtuse, sometimes apically setulose. Stolons filiform, terete, glabrous, up to 30 cm long, 0.4-0.6 mm thick, sparsely branched, markedly dimorphic; some green, growing on the surface of the substrate or suspended or floating; others without chlorophyll and ± buried in the substrate. Air shoots not seen. Leaves very numerous, polymorphic; those on stolons above the substrate complanate, imbricate, approximately circular in outline, 0.1-2 cm long, palmato-dichotomously divided into up 15 segments, the ultimate segments flattened, narrowly linear, with apex obtuse, 0.1-0.7 mm wide, the margin typically entire and bearing throughout its length, up to 20 short setulae, or sometimes (on leaves produced at the beginning and end of the growing season) sparsely denticulate, the teeth very small acute, each with an apical, solitary setula or fascicle of up to 4 setulae; setulae 3-10 times as long as their tooth (Thor 1988); the leaves on stolons beneath the substrate fewer and ultimate segments at the base and near the apex. Traps rather few, lateral on the segments, absent or usually so on those above substrate, 1-3 normally present on those below substrate, ovoid, stalked, 1.5-4 mm long, the mouth lateral, with 2 long, much branched, setiform, dorsal appendages and a few latera, simple setae, the internal glands 2- and 4-armed, the arms narrowly cylindrical, with apex subacute, up to 14 times as long as wide, the quadrifids with two pairs typically both parallel, or sometimes slightly divergent. Inflorescence erect, emergent, 10-20 cm long; peduncle filiform, terete, glabrous, straight, 0.5-0.8 mm thick. Scales 2 or 3, ± equally spaced on the peduncle, similar to the bracts. Bracts basifixed, broadly ovate or ovate-deltoid, conspicuously auriculate at the base, with apex acute, several-nerved, 3 mm ca. long. Bracteoles absent. Flowers 2 or 3, the raceme axis short; pedicels erect, filiform, terete, 0.5-1.5 cm long. Calyx lobes subequal, 3-4 mm long, ovate, the upper lobe with apex acute, the lower lobe shorter, with apex obtuse, shortly bifid or truncate. Corolla yellow, 1-1.6 cm long; upper lip broadly ovate with apex rounded; lower lip transversely elliptic, the base with a

prominent rounded swelling, the apex rounded; palate glabrous; spur subulate to a lesser extent ventrally, glandular, Filaments curved, 2 mm ca. long, the anther thecae ± confluent. Ovary globose, glandular; style relatively long; stigma lower lip circular, ciliate, the upper lip much smaller, deltoid with apex acute or bifid. Capsule globose, 2.5-3 mm in diameter, circumscissile. Seeds rare, in the few brief descriptions and illustrations (e.g. by Hayne himself) similar to those of *U. minor*, but a little longer. Turions globose to ovoid, 3-6 mm long, up to 4 mm wide, curved on one side, hairy, but barely visible at naked eye, because covered by mucilage; light green or greyish green (Meister 1900).

Pollen grains suboblate, radially symmetric, subisopolar and zonocolporate with (11)12-15(16) colpori. The profile of the colpus margin is regular and there are sporadic granules on the colpus membrane; anastomosing colpori are occasional near the polar region. The polar regions are slightly different in size: one pole has a wider surface and as a consequences a greater number of fossulae than the other pole. The ornamentation of the tectum is psilate on the mesocolpium thickening at the equatorial region and fossulate on the apocolpium. In this species, the fossulate pattern of the ornamentation has a wide extension as it involves a part of the mesocolpium and ends just before the equator. Therefore, the whole ornamentation is psilate-fossulate (Beretta et al. 2014).

Chromosome number $2n = 44$ (Casper & Manitz 1975).

Utricularia intermedia has a circumboreal distribution, occurring in North America, Asia and central-northern Europe.

Living in bogs and marshes in shallow water, or sometimes in streams, lakes and ponds in deep water (but never flowering in such situations) from sea level to high altitudes, flowering in the warmest part of the year.

Utricularia ochroleuca **R.Hartm.**, Botaniska Notiser 1857: 30 (1857).

Protologue: *U. ochroleuca n. sp. – Foliis distichis, laciniis planis plus minus parce vesiculiferis, labio corollae superiore palatum inflatum bis superante, calcare brevi conico a labio inferiore descendente. Jul. Aug. Hab in aqua stagnante paludum. Hucusque lecta in paludosis haud procul a lacu Tönshammarsjön par. Skog Helsingiae australis. Herba tota gracilis ac tenera. Rami foliiferiundique protensi, 5-8'' longi, simplices vel ramulosi; gemmae, folia future anni continents, globosae et magnitudine illarum U. minoris, quae gemmae ne ramis nudis vesiculiferis quidem desunt; folia*

conserta vel subdistantia, disticha, primo tripartita deinde dichotoma, laciniis planis spinuloso-denticulatis acutis nervo plus minus distincto instructis; vesiculae partim inter lacinias foliorum sparsae, partim ad ramos nudos adfixae; scapus fere digitalis, 2-5-florus una cum bracteis, pedunculis calycibusque rufo-fuscescentis coloris; corolla pallide flava vel ochroleuca, labio superiore palato duplo longiore, integro striato, labio inferiore rotundato, lateribus deflexo, calcare descendente, conico-obtuso, rufescente, labio inferiore semper multo breviore; stamina semilunaria compressa, apice latiora, antheris ovatis liberis; stylus brevis teres, stigmate lanceolato; pedunculi fructiferi patentes. Praecedenti affinis, a qua differt: herba tota multo tenuiore; vesiculis non solum ramis nudis, sed etiam foliis adhaerentibus; scapo rufescenti-brunneo; colore corollae pallidiore; longe alia forma, coloreet directione calcaris. U. intermedia enim vesiculis inter lacinias fol. onimno destituta praebet scapum, bracteas calycesque semper laete viridia et calcar (subulatum, labio inferiori adpressum) ejusdem coloris ac corolla et saepissime ejusdem longitudinis ac labium inferius.

Lectotype (designated by Thor 1988: 221): Sweden, Hälsingland, Hemstanäs, vid Tönshammarsjön, *Hartman* s.n. (GB! see Fig. 3).

Small perennial usually affixed aquatic. Rhizoids rare, 0-1, 20-40 mm long, 0.2-0.7 mm thick, bearing numerous short dichotomously divided branches, the ultimate segment minutely papillose, shortly cylindrical, acute at the apex, sometimes ending with one bristle each, 1-4 mm long. Stolons filiform, terete, glabrous, up to 25 cm long, 0.3-0.5 mm thick, sparsely branched, markedly dimorphic, some green, growing on the surface of the substrate or suspended or floating, others without chlorophyll and ± buried in the substrate. Air shoots not seen. Internodes 2-6 mm ca.; leaf segments flat, subulate, with a sometimes indistinct mid-rib, approximately circular in outline, 0.2-1.5 cm long, palamto-dichotomously divided into up to 20 segments ca., the ultimate segments flattened, narrowly linear with apex acute, 0.1-0.5 mm wide, the margin always sparsely denticulate, the teeth (0)1-3(5) in number, furnished with 1 or rarely 2 setulae each; setulae 0.3-7 times ca. as long as their tooth. Leaves on stolons beneath the substrate fewer and ± reduced to a single elongate segment with a few, very much reduced short segments at the base and near the apex. Traps rather few, lateral on the segments, usually 1 or sometimes more present on those above the substrate, 1-3 normally present on those below the substrate, ovoid, stalked, 1-3 mm long, the mouth lateral with 2 long, much

branched, setiform, dorsal appendages and a few lateral simple setae, the internal glands 2- and 4-armed, the arms narrowly cylindrical-subulate, with apex obtuse or subacute, the bifids up to 140 μm long and up to 12 μm wide, the quadrifids with both pairs divergent with an included angle between shorter arms of 115°-230° and between longer arms of 19°-52° (Thor 1988). Inflorescence erect, emergent, 8-15 cm long; peduncle filiform, terete, glabrous, straight, 0.5-0.8 mm thick. Scales 3-4, ± equally spaced on the peduncle, similar to the bracts. Bracts basifixed, broadly ovate, with apex obtuse to acute and base conspicuously auriculate, 1-several-nerved, 2.5 mm ca. long. Bracteoles absent. Flowers 1-4 (Thor 1988), the axis short or slightly elongate; pedicels erect, spreading post-anthesis, filiform, terete, 5-8 mm long. Calyx lobes slightly unequal, ovate 3-4 mm long, the upper lobe with apex acute, the lower lobe shorter and broader, with apex shortly bifid. Corolla pale yellow, 1-1.5 cm long; upper lip broadly ovate with apex rounded; lower lip at first almost flat, later with deflexed margin (Thor 1988), palate glabrous; spur conical, sometimes with the distal part shortly cylindrical, with apex ± acute, usually about half as long as the lower lip, the internal surface dorsally, and to a lesser extent ventrally, glandular. Filaments curved, 2 mm ca. long, the anther thecae ± distinct. Ovary broadly ellipsoid; style short; stigma lower lip circular, ciliate, the upper lip much smaller, semicircular. Capsule very rare, seen only once, globose, 2 mm in diameter. Turions not seen (Thor 1988).

Pollen grains oblate spheroidal, radially symmetric, subisopolar and zonocolporate with (11)12-14(15) colpori. Asymmetric, spiraperturate and micropollen grains have been frequently observed, especially in the population from Beuren (Germany). The profile of the colpus margin is regular and there are sporadic granules on the colpus membrane; anastomosing colpori are very frequent near the polar region in normal grains and on the whole surface on malformed grains. The polar regions are slightly different in size: one pole has a wider surface and as a consequence a greater number of fossulae than the other pole. The ornamentation of the tectum is psilate with sporadic perforations (diameter 0.1–0.3 μm) on the mesocolpium (thickened on the equator), the number of perforations increases towards the apocolpium and the ornamentation becomes fossulate. Therefore, the whole ornamentation is psilate-fossulate (Beretta et al. 2014).

Chromosome number $2n$ = ca. 40 (Reese 1952), $2n$ = 44, 46, 48 (Casper & Manitz 1975). Both *U. ochroleuca* and *U. stygia* have a circumboreal distribution and they often have been confused with each other (see Thor 1988, Fleischmann & Schlauer 2014), so that it

is difficult to exactly outline the area of occurrence of these species. In Europe, *U. ochroleuca* mostly occurs in central Europe (France, Germany, Poland and Czech Republic) and in Scandinavia (Sweden and Finland).

Living in bogs and marshes in shallow water, sometimes in deeper water in streams and lakes but not flowering in such situations, mostly at low, but sometimes at higher altitudes, to 2800 m. According to Thor (1988), it grows usually in slightly eutrophic and semi-rich habitats and it does not thriven when the field layer becomes too luxuriant. According to Kosiba & Stankiewicz (2007) it prefers eutrophic shifting to dystrophic water microhabitats. *Utricularia ochroleuca* is usually growing with *U. intermedia* and *U. minor*, and only in Czech Republic (South Bohemia), with *U. stygia* (Thor 1988, Płachno & Adamec 2007). Flowering in the summer.

Neuman (1900) considered *U. ochroleuca* as morphologically intermediate between *U. intermedia* and *U. minor* and for this reason, he postulated its possible hybrid origin from a cross between these two species. Alternatively, Taylor (1989) considered this species as a vegetative apomict derived from *U. intermedia*, based on the different dysploid chromosome numbers found.

Utricularia stygia **Thor**, Nordic Journal of Botany 8: 219 (1988).

Protologue: *Differt ab U. ochroleuca segmentis foliorum 0.19-0.44 mm latis margine 2-7 dentato. Antennae quadrifidae brachia longiora (79-)94-131(-168) × 9-12 μm; brachia breviora (47-)62-88-(-116) × 9-12 μm; angulus inter brachia longiora (16°-)26°-56°(-90°); angulus inter brachia breviora (30°-)52°-97°(-140°); angulus inter brachia longiora et breviora (80°-)106°-139°(-175°). Flores lutei leviter rubelli. Labium inferius planum vel margine leviter sursum versus adscendenti, 9-11 × 12-15 mm.*

Holotype: Sweden, Södermanland, St. Malm par., 2.5 Km ca. SE of Strångsjö, N part of the marsh Blomsterskärret, 300 m ca. E of road 55, 58°53' N 16°13'E, 19 Jul 1986 *Thor* 6581 (S! see Fig. 4, isotypes C, GH, H, K, LE, MT, O, UPS).

Small perennial usually affixed aquatic. Rhizoids rare, 0-1, 10-40 mm long, 0.2-0.7 mm thick, branched, the ultimate segments acute at the apex, ending with a setula each, 1-4 mm long. Stolons filiform, terete, glabrous, up to 20 cm long, 0.3-0.5 mm thick, sparsely branched, markedly dimorphic, some green, growing on the surface, of the substrate or suspended or floating, others without chlotophyll and ± buried in the substrate. Air shoots

not seen. Internodes 2-5 mm ca.; leaf segments flat, subulate, with a sometimes indistinct mid-rib, approximately circular in outline, 0.2-0.45 mm wide, the margin always sparsely denticulate, the teeth (2)3-6(7) in number, furnished with 1-2 setulae each; setulae 0.3-7 times ca. as long as their tooth. Leaves on stolons beneath the substrate fewer and ± reduced to a single elongate segment with a few, very much reduced short segments at the base and near the apex. Traps usually both on green and colourless shoots but most traps on colourless shoots or rarely with traps on only colourless shoots, 0-1 per leaf, ovoid, stalked, 1-3 mm long, the mouth lateral with 2 long much branched, setiform, dorsal appendages and a fewer lateral, simple setae, the internal glands 2- and 4-armed, the arms narrowly cylindrical-subulate, with apex obtuse or subacute, the bifids up to 150 μm long and up to 12 μm wide, the quadrifids with both pairs divergent with an included angle between longer arms of 16°-90° and between shorter arms of 30°-140°. Inflorescence erect, emergent, 5-15 cm long; peduncle filiform, terete, glabrous, straight, 0.5-0.8 mm thick. Scales and bracts probably as in *U. ochroleuca*. Bracteoles absent. Flowers 1-4; pedicels straight, 3-6 mm ca. long, after flowering recurved, not prolonged. Calyx lobes slightly unequal, ovate 2-3 mm long, the upper lobe with apex acute, the lower lobe shorter and broader, with apex shortly bifid. Corolla yellow with a reddish tinge; upper lip 8 mm long; lower lip flat or margins slightly curved upwards, 7-9 mm long and 12 mm ca. wide (Fleischmann & Schlauer 2014); spur conical, tapering upwards, up to 7 mm long, directed downwards from the lower lip at an acute angle and with internal glands on both the abaxial and adaxial side. Gynoecium and androecium probably as in *U. ochroleuca*. Capsule not seen. Turions globose to ovoid, 5-15 mm in diameter, with tiny hairs (Fleischmann & Schlauer 2014).

Pollen grains from ellipsoidal to spheroidal, asymmetric, heteropolar and often malformed. Irregular anastomosing colpori are very frequent and the grains appear often spiraperturate; a large number of gigapollen grains have been observed. The rare normal grains are zonocolporate with (10)12-14(15) colpori; the tectum is nearly continuous and the ornamentation is psilate on themesocolpium (thickened on the equator) and fossulate on the apocolpium (Beretta et al. 2014).

Chromosome number unknown.

Utricularia stygia is present in the same countries of *U. ochroleuca* (except Poland), but it extends also in British Islands, and southwards in Switzerland and northern Italy (Taylor 1989, Płachno & Adamec 2007).

Living in stagnant, shallow water in marshes and on quagmires. It is only seen in oligotrophic habitats. *Utricularia stygia* is usually found in deeper water than *U. intermedia* and *U. ochroleuca* and it often grows with *U. intermedia* and *U. minor* and only in South Bohemia it was found with *U. ochroleuca* (Płachno & Adamec 2007). The altitude ranges from sea level up to 1650 m ca (Thor 1988, Schlosser 2003). Flowering in the summer.

Parallel to *U. ochroleuca*, *U. stygia* might probably represent a hybridogenic taxon, derived from a putative original cross between a species of *U. intermedia* aggr. and a species of *U. minor* aggr. (Schlosser 2003, Płachno & Adamec 2007). The hypothesis of Taylor (1989) to consider *U. ochroleuca* as a vegetative apomict derived from *U. intermedia* could be in the same way applied to *U. stygia*.

Because the confusion with *U. ochroleuca* and the subsequent difficulty to delimit its distribution, *U. stygia* lacks an adequate assessment of its conservation status (i.e. extinction risk categories of the IUCN protocol; IUCN 2014) also at regional levels. Indeed, it is reported as DD in Great Britain (Cheffings et al. 2005), not evaluated in two countries where it occurs with certainity as Czech Republic (Holub & Procházka 2000) and Switzerland (Moser et al. 2002). In France and in Italy it has been assessed to VU (UICN France et al. 2012) and CR extinction risk categories, respectively (Beretta & Tassara 2010).

Utricularia minor aggr.

Utricularia bremii **Heer** in Koelliker, Verzeichniss der Phanerogamischen Gewächse des Kantons Zürich: 142 (1830).

Protologue: *U. nectario brevissimo, gibbo, labio superiori integro, palato paulo longiore; fauce aperta. Habitus Utriculariae minori similis. Caulis pedalis et ultra, scapus longior, 6.8 florus. Folia inferne saepe 6''' distantia, non omnia utriculata, breviora, in ramos duos principals divisa, laciniae magis divergentes, utriculi minus longe pedicellati. Flores duplo feremajores, lutei, ut in perius integrum vel subapiculatum, palate paulo longius, elongate-ovatum; inferius latius, brevius magisque obtusus. Nectarium labio inferiori paulo brevius, lateribus bistriatum. Germen ovato-rotundatum. – Cum U. intermedia florum colore, labio superiori integro convenit; maxime autem nectario multo breviori, fauce aperta, floribus minoribus, foliis omnibus aequalibus differt.*

U. Bremii. Heer. In Torfmooren selten. Am Katzensee, Bremi. Heer. Köll.

Lectotype (designated by Taylor 1989: 613): Switzerland, Katzensee, *Bremi* s.n., 26 June 1836 (not located). Note: in accordance with Art. 9.23 of the ICN (McNeill et al. 2012), only for lectotypification after 1 January 1990 the specification of the herbarium where the type is conserved is needed. In his monograph, Taylor (1989) designated as type the aforementioned collection. Hence, this can be considered as a first-step lectotypification (Art. 9.17, Ex. 12 of the ICN, McNeill et al. 2012). A specimen corresponding to *U. bremii*, collected in the site indicated in the protologue (Katzensee) by J. Bremi and corresponding to the date indicated by Thor does exist in Zürich (ZT! see Fig. 5) and could be subject to a second-step lectotypification (Art. 9.17, Ex. 12 of the ICN, McNeill et al. 2012).

Small, perennial, affixed or suspended aquatic. Rhizoid absent. Stolons filiform, terete, glabrous, up to 25 cm long, 0.3-0.5 mm thick, sparsely branched, ± dimorphic; some green, on the surface of the substrate or suspended or floating; others without chlorophyll and ± buried in the substrate. Air shoots not seen (Thor 1988). Leaves very numerous, polymorphic; those on the stolons above the substrate circular to ovate in outline, 0.5-2 cm long, palmato-dichotomously or pinnato-dichotomously divided into rather numerous segments, the ultimate segments up to 50, flattened, filiform to linear, 0.1-0.5 mm wide, the margins entire or distally sparsely denticulate, the teeth apically setulose, with apex acute, setulose; leaves of stolons beneath the substrate ± reduced to 1 or 2 capillary, primary segments, bearing a few reduced, usually present, but few on those above substrate, more numerous and larger on those below substrate, ovoid, stalked, 1-2 mm long, the mouth lateral with 2 long, much branched, setiform, dorsal appendages and a few lateral, simple setae, the internal glands 2- and 4-armed, the arms narrowly cylindrical with apex subacute, up to 100 μm long and up to 15 times as long as wide, the quadrifids with the longer pair parallel or slightly divergent and the shorter pair very widely divergent to slightly reflexed, with an included angle of 180° to 200°. Inflorescence erect, emergent, 5-50 cm long; peduncle filiform, terete, glabrous, straight, up to 1 mm thick. Scales 2-4, ± equally spaced on the peduncle, similar to the bracts. Bracts basifixed, broadly ovate, with the base conspicuously auriculate and with the apex obtuse, several nerved, 1.5-2 mm long. Bracteoles absent. Flowers 2-14, the raceme axis ± elongate; pedicels erect at the anthesis, later spreading and distally decurved, filiform, terete, 0.4-

1.5 cm long. Calyx lobes subequal, 2-3 mm long, ovate, the upper lobe with apex obtuse, the lower lobe slightly smaller, with apex very shortly bifid. Corolla yellow, 8-10 mm long, about as long as wide; upper lip broadly ovate with apex retuse; lower lip limb transversely elliptic or circular with apex rounded; palate elongate with a raised, distally glandular, emarginated rim; spur shortly conical, obtuse, in lateral view about as wide as long, the internal surface ventrally densely glandular. Filaments curved, 1.5 mm ca. long, the anther thecae subdistinct. Ovary globose; style relatively long; stigma lower lip semicircular, ciliate, with apex reflexed, the upper lip much smaller, deltoid, with apex bifid. Capsule and seeds not seen. Meister (1900) reported that only one time in July 1899 he reached to find a young fruit near Dübendorf and that of 96 herbarium he investigated, accounting for about 500 flowers, only one was fertile. Adamec (2002) reported the occurrence of seeds in ex situ cultivated plants originally collected in northern Russia (Lake Onega).

Pollen grains are from ellipsoidal to spheroidal, asymmetric, heteropolar and they often appear deformed. Irregular anastomosing colpori are very frequent on the whole surface and the grains often appear spiraperturate. Few normal grains have been observed and they are zonocolporatewith 10-13(14) colpori. The tectum is nearly continuous and the ornamentation is perforate (perforations ~ 0.1–0.3 μm) (Beretta et al. 2014). Turions globose, vivid green, not hairy, ca. 4 mm in diameter (Meister 1900).

Chromosome number $2n = 36$ (Rahman et al. 2001).

Utricularia bremii is endemic to Europe, mostly in the central part of the continent (Belgium, France, Germany, Czech Republic, Poland, Switzerland and northern Italy; Krajewski & Plachno 2015).

In bogs in shallow water or sometimes (not flowering) in deeper water at low and medium altitudes. Fleischmann & Schlauer (2014) reported the species secondarily also on peaty soils and sandpits in abandoned fishponds. According to Krajewski (2015), the Polish populations prefer oligotrophic and mesotrophic habitats, fully insulated areas with scattered vegetation. This confirmed what already found in Slovakia by Dite et al. (2013). Interestingly, *U. bremii* often co-occurs with *U. australis*, e.g. in Slovakia, Bavaria and Italy (Beretta et al. 2011, Dite et al. 2013, Fleischmann & Schlauer 2014, personal observations). Flowering rarely, in the summer.

Utricularia bremii could probably represent a vegetative apomict derived from *U. minor* (Taylor 1989), or alternatively the product of the hybridization between a species of *U.*

intermedia aggregate and *U. minor*, similarly to other mostly sterile European species as *U. ochroleuca* and *U. stygia*.

Despite assessed as DD in the IUCN European Red List, *U. bremii* is rare and threatened in all the countries of occurrence. Indeed, at national level is reported as endangered (EN) in Switzerland (Moser et al. 2002), critically endangered (CR) in Italy (Beretta et al. 2011), Germany (Ludwig & Schnittler 1996), Austria (Niklfeld & Schratt-Ehrendorfer 1996, Fischer et al. 2008), Hungary (Király 2007), Czech Republic (Grulich 2012) and Slovakia (Dítě et al. 2013).

***Utricularia minor* L.**, Species Plantarum: 18 (1753).

Protologue: *Utricularia nectario carinato. [...] Habitat in Europae fossis rarius. Nectarium obsoletum, deorsum spectans; faux absque gibbo palate, pervia & hians.*

Lectotype (designated by Casper in Rechinger 1969: 2): Herb. Linn. no. 34.3 (LINN!) http://linnean-online.org/271/.

Small perennial, usually affixed, aquatic. Rhizoids absent. Stolons filiform, terete, glabrous, up to 30 cm long, 0.1-0.5 mm thick, sparsely branched, ± dimorphic; some green, on the surface of the substrate or suspended or floating; others without chlorophyll and ± buried in the substrate. Air shoots not seen (Thor 1988). Leaves very numerous, polymorphic, those on stolons above the substrate approximately semicircular in outline, 0.2-1.5 cm long, palmato-dichotomously divided into 7-22 segments, the ultimate segments flattened, filiform to linear, 0.1-1 mm wide, the margins entire or sometimes very sparsely denticulate but the teeth not or only microscopically setulose, the apex acute with or without a microscopic setula, the leaves on the stolons beneath the substrate ± reduced to one or two elongate primary segments with a few very much reduced very short further segments at the base and apex. Traps lateral on the segments, rather few or absent on those above substrate, more numerous and larger on those below substrate, ovoid, stalked, 0.8-2.5 mm long, the mouth lateral with 2 long, much branched, setiform, dorsal appendages and a few lateral, simple setae, the internal glands 2- and 4-armed, the arms subulate, tapering to a rounded apex, up to 100 μm long up to 14 times as long as wide, the quadrifids with the longer pair subparallel or divergent of an included angle of up to 25° ca., the shorter pair reflexed, with an included angle of 270° to 300°. Inflorescence erect, emergent, 2.5-25 cm long; peduncle filiform, terte, glabrous, straight,

0.5-0.8 mm thick. Scales 2-4, ± equally spaced on the peduncle similar to the bracts. Bracts basifixed, broadly ovate, with the base conspicuously auriculate, and the apex acute or obtuse, 1-several nerved, Bracteoles absent. Flowers 2-6, the raceme axis initially short, elongating with age; pedicels erect at the anthesis, later spreading and distally decurved in fruit, filiform, terete, 4-8 mm long. Calyx lobes subequal, 2-3 mm long, broadly ovate, the upper lobe with apex sub-acute, cucullate, the lower lobe somewhat smaller, with apex narrowly truncate. Corolla lemon-yellow, 6-8 mm long, usually longer than wide; upper lip ovate or ovate-oblong with apex retuse; lower lip limb broadly obovate with apex rounded or retuse, the lateral margins curved downwards; palate elongate, with a raised marginal rim, distally narrowed and glandular; spur saccate or obtusely broadly conical, in lateral view wider than long, the internal surface densely glandular. Filaments curved, 1.5 mm ca. long, the anther thecae subdistinct. Ovary broadly ellipsoid; style relatively long; stigma lower lip broadly ovate, ciliate, with apex reflexed, the upper lip much smaller, deltoid, with apex acute or 2-3-fid. Capsule globose, 2-3 mm in diameter, circumscissile. Seeds lenticular-prismatic, 1 mm ca. wide and ⅔ ca. as long, scarcely winged on the angles, the testa cells approximately isodiametric.

Pollen grains prolate spheroidal, radially symmetric, isopolar and zonocolporate with (10)11-14(15) colpori. The profile of the colpus margin is regular and there are sporadic granules on the colpus membrane; anastomosing colpori are rare. The ornamentation of the tectum is psilate on the thickened equator, perforate between the equator and the poles (perforation diameter 0.1–0.3 μm) and finely fossulate on polar regions. Therefore, the whole ornamentation is psilate–finely fossulate (Beretta et al. 2014). Turions globose, vivid green, not hairy, lesser than 3 mm ca. in diameter (Meister 1900).

Chromosome number $2n$ = 36-40 (Reese 1952), $2n$ = ca. 40 (Löve & Löve 1956), $2n$ = 44 (Casper & Manitz 1975).

Utricularia minor has a circumboreal distribution, extending southwards to the Himalaya, and in Europe occurs throughout the continent, but it is rare in the Mediterranean area.

Living in bogs and marshes in shallow water, less commonly, and not flowering, in deeper water. According to Thor (1988), the species is usually found in oligotrophic, rarely in eutrophic, habitats, often along with *U. intermedia*. Populations from Poland (Kosiba & Stankiewicz 2007) are reported to be mainly adapted to dystrophic waters, along with *U. intermedia*. Fleischmann & Schlauer (2014) stated that the Bavarian populations inhabit mostly acid waters, but it can be found also on sparse vegetation context, in waterbodies

fed by calcareous sources. From sea level to very high altitudes especially in the southern part of its range in Asia. Flowering during the warmest season.

Utricularia vulgaris **aggr.**

Utricularia australis **R.Br.**, Prodromus florae Novae Hollandiae et Insuale Van diemen: 430 (1810).

Protologue: *U. australis, scapo paucifloro, labiis indivisis: inferiore duplo latiore quam longo, calcari adscendenti anticè plano subtus carinato. (J. D[1]) v.v.*

(Lectotype designated by P. Taylor 1973: 20): Australia, New South Wales, between Hawksbury and Paramatta, *R. Brown* s.n. (BM! see Fig. 6).

Medium sized to large perennial, suspended aquatic. Rhizoid usually present, few, filiform, a few cm long, 0.5 mm ca. thick, bearing numerous short, dichotomously divided branches with narrowly ovoid, papillose, apically setulose, ultimate segments. Stolons light green to dark green (Thor 1988), filiform, branched, up to 50 cm long or longer, 0.3-1 mm thick, terete, glabrous, the internodes 0.5-2 mm long. Air shoots rare, 2-15 cm long (Thor 1988). Leaves very numerous, 1.5-4 cm long, divided from the base into 2 ± equal, primary segments, each ± pinnately divided, the secondary segments dichotomously divided into further segments, the ultimate segments capillary, somewhat flattened, apically and laterally minutely setulose, the lateral setulae each arising from the apex of a short ± acute tooth. Teeth (4)6-8(10), furnished with 1-2 setulae each; setulae 2-10 times ca. as long as their tooth (Thor 1988). Traps dimorphic, usually moderately numerous, arising laterally from the secondary to penultimate segments, also at the base of the primary segments, the lateral traps ovoid, stalked 0.5-2.5 mm long, the mouth lateral, with 2 dorsal, setiform, simple or branched appendages and usually with further, simple or branched appendages, the internal glands of all traps 2- and 4-armed, the arms subulate, with apex subacute, up to 70 μm long up to 12 times as long as wide, the quadrifids with longer pair subparallel to divergent with an included angle of up to 45°, the shorter pair typically divergent with an included angle of 180° ca., but the angle vary from 30° to 200°. Inflorescence weakly erect, emergent, 10-30(100) cm long; peduncle filiform,

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[1] In brackets, J. stands for Port Jackson and D. for Van Diemen Island.

terete, glabrous, 1-2 mm thick, at first straight, becoming flexuous. Scales 1(3), always present in the upper half of the peduncle, similar to bracts. Bracts basifixed, approximately circular, the base auriculate, the apex rounded or obscurely tridentate, many nerved, 3-5 mm long. Bracteoles absent. Flowers 4-10, the raceme axis initially short, elongating with age; pedicels filiform, terete 1.5-3 cm long, erect at the anthesis, post-anthesis spreading or, when fruit is produced, decurved. Calyx lobes slightly unequal, ovate, 3-4 mm long, the upper lobes with apex rounded, the lower lobe with emarginated. Corolla yellow with the basal swollen part of the lower lip much darker and with reddish brown lines and spots, 1.2-2 cm long; upper lip very broadly ovate, with apex retuse; lower lip limb reniform or transversely elliptic, the base with a very prominent swelling, the distal part ± flat, up to 1.8 cm wide, with apex rounded or retuse; palate glabrous; spur broadly conical, with apex obtuse, slightly curved, considerably shorter than the lower lip, covered, inside the whole of the distal half, with regularly distributed, subsessile glands. Filaments curved, 2 mm ca. long, the anther thecae distinct. Ovary globose, densely covered with sessile glands; style distinct; stigma lower lip semicircular, ciliate, the upper lip very short or obsolete. Capsule extremely rare, globose, 4 mm ca. in diameter, circumsessile. Seeds prismatic, 4-6 angled, 0.5 mm ca. long and wide, narrowly winged on all the angles, the testa cells elongate with the anticlinal boundaries raised and the periclinal walls tubular, smooth.

Pollen grains oblate spheroidal, radially symmetric, subisopolar and zonocolporate with (10)11-15(16) colpori. The profile of the colpus margin is regular and there are sporadic granules on the colpus membrane; anastomosing colpori are occasional near the polar region. The polar regions are slightly different: one pole has a wider surface and a more complex pattern of fossulae which are also more numerous if compared with the other pole. On mesocolpium (thickened on the equator), the ornamentation of the tectum is psilate with sporadic perforations (diameter 0.1-0.3 μm). The number of perforations increases towards the apocolpium where the ornamentation becomes fossulate. Therefore, the whole ornamentation is psilate-fossulate (Beretta et al. 2014).

Turions globose to ovate, 4-15 mm long (Thor 1988).

Chromosome number $2n = 36\text{-}40$ (Reese 1952), $2n = 36, 38, 40, 44$ (Casper & Manitz 1975).

According to Taylor (1989), *U. australis* is widely distributed in 4 continents, occurring all over Europe (except the far north), in temperate and tropical areas of Asia and Africa, in New Zealand, and Australia, where the species was originally described.

Living in lakes, pools, rivers backwaters, ditches in still or slowly flowing water, mostly at low altitude but ascending to high altitudes in the tropics. According to Thor (1988), the Nordic populations prefer habitats more or less oligotrophic, while Kosiba and Stankiewicz (2007) reported for Polish populations habitats characterized by waters shifting from eutrophic to dystrophic conditions. Fleischmann & Schlauer (2014) reported that Bavarian populations inhabit oligotrophic waters, typically neutral or slightly acid, rarely slightly alkaline. Flowering in Europe during the latter part of the summer, in the tropics in the wet season and in Australia in December to February.

According to Kameyama et al. (2005), this species originated from an asymmetric hybridization event involving *U. tenuicaulis* (probably female parental species) and *U. macrorhiza* (probably male parental species), in North Japan. Recurrent hybridizations and clonal propagation were also demonstrated to be crucial for its radiation as well as hybrid superiority (vigour) in certain environmental conditions, since hybrids and parental species does not co-occur. In addition, long dispersal of clonal offspring might have ensured the maintenance of hybrid populations (Kameyama & Ohara 2006).

***U. vulgaris* L.**, Species Plantarum: 18 (1753).

Protologue: *Utricularia nectario conico […]. Habitati in Europae fossis paludibus profondioriubs. Nectarium subulatum, longitudine labii inferioris, cui approximatum; faux clausa palato gibbo. Plant nobis duplex: altera MAJOR a Rivino delineata, calyce postice rotundato. Altera MINOR calyce postice transverso, & quasi truncato; crescunt conjunctim.*

Lectotype (designated by Taylor 1964: 81): Herb. Linn. no. 34.2 (LINN!) http://linnean-online.org/270/.

Medium sized to large, perennial, suspended aquatic. Rhizoids usually present, few, filiform, a few cm long, 0.5 mm ca. thick, bearing numerous short, dichotomously divided branches with narrowly ovoid, papillose, apically setulose, ultimate segments. Stolons dark green to brownish (Thor 1988), filiform, branched, up to 1 m long or longer, 0.5-1.5 mm thick, terete, glabrous, the internodes 0.5-2 cm long. Air shoots rare, 2-15 cm long

(Thor 1988). Leaves very numerous, 1.5-6 cm long, divided from the base into 2 unequal primary segments, each ± pinnately divided into further segments, the ultimate segments capillary, somewhat flattened, apically and laterally minutely setulose, the lateral setulae each arising from the apex of a short ± acute tooth or arising directly from the leaf margin. Teeth (4)6-8(10), furnished with 1-2 setulae each 3-12 times as long as their tooth (Thor 1988). Traps dimorphic, usually moderately numerous, arising laterally from the secondary to penultimate segments, also at the base of the primary segments; lateral traps ovoid, stalked, 1.5-5 mm long, the mouth lateral, with 2 dorsal, setiform, simple or usually branched appendages and usually with further simple lateral setae; basal traps ovoid, stalked, the mouth basal, naked or with 2 very short, setiform, simple appendages, the internal glands of both kinds 2- and 4-armed, the arms subulate with apex rounded, up to 70 μm long and up to 10 times as long as wide, the quadrifids with the longer pair parallel or divergent with an included angle of 90-120°. Inflorescence erect, emergent, 10-25 cm long; peduncle filiform, terete, glabrous, straight, 1-2.5 mm thick. Scales 2-4(5), always present, mostly in the upper half of the peduncle, similar to the bracts. Bracts basifixed, broadly ovate, the base ± cordate or shortly auriculate, the apex acute to obtuse, many nerved, 3-5 mm long. Bracteoles absent. Flowers 6-12, the raceme axis initially short, elongating with age; pedicels filiform, terete, 0.6-1.2 cm long, erect at the anthesis, strongly decurved in fruit. Calyx lobes slightly unequal, ovate 3.5 mm long, glandular, the upper lobe with apex acute or subacute, the lower lobe shorter, with apex obtuse, emarginated. Corolla yellow with reddish brown streaks on the swollen basal part of the lower lip, 1.4-1.9 cm long; upper lip very broadly ovate with apex retuse; lower lip limb very broadly ovate, the base with a very prominent swelling, the lateral margins strongly deflexed, the apex retuse, ca. 1.5 cm wide when flattened; palate covered, inside the distal half, with short hairs and stipitate glands; spur shorter than the lower lip, with a broad, conical base and a narrow cylindrical or narrowly conical, acute apex, the distal 2/3, when viewed from the side, with the ventral surface typically straight, sometimes slightly concave or convex, with internal glands on the dorsal surface only. Filaments curved, 2 mm ca. long, the anther-thecae subdistinct. Ovary globose, densely glandular; style distinct; stigma lower lip approximately circular, ciliate; upper lip very short, truncate. Capsule globose, 5 mm ca. in diameter, circumscissile. Seeds prismatic, 4-6 angled, 0.6 mm ca. wide and about half as high, the testa cells slightly elongate on the distal surface, more elongate on the lateral and proximal surface, the anticlinal walls almost straight to

markedly sinuate, slightly raised, microscopically granulose, the periclinal walls tubular, microscopically granulose.

Pollen grains prolate spheroidal, radially symmetric, subisopolar and zonocolporate with 15-19 colpori. The profile of the colpus margin is regular and there are sporadic granules on the colpus membrane; anastomosing colpori are occasional near the polar region. The polar regions are slightly different in size: one pole has a wider surface and as a consequence a greater number of fossulate than the other pole. The ornamentation of the tectum is psilate with sporadic perforations (diameter 0.1-0.3 μm) on the mesocolpium, the number of perforations increases towards the apocolpium and the ornamentation becomes fossulate. In this species the fossulate pattern of the ornamentation has a wide extension because it involves a part of the mesocolpium and ends just before the equator. Therefore, the whole ornamentation is psilate-fossulate.

Turions globose to ovate, 4-15 mm long (Thor 1988).

Chromosome number $2n = 36-40$ (Reese 1952), $2n = 40$ (Löve 1954), $2n = 44$ (Casper & Manitz 1975).

Utricularia vulgaris occurs in North Africa, Asia (temperate areas, in Siberia and Tibet) and Europe, all over the continent (except Arctic, rare in the south).

Living in lakes, pools, ditches and river backwaters, in still or slowly flowing water, usually at low altitude, but ascending to considerable altitudes in the southern parts of its range. According to Thor (1988), the Nordic populations can be found both on oligotrophic and eutrophic waters, and this was confirmed by Kosiba & Stankiewicz (2007), reporting a wide range of water conditions for Polish populations, but preferably in eutrophic ones. Even Bavarian populations are reported in both eutrophic and oligotrophic waters, but usually in neutral or slightly alkaline waters. Flowering in the summer.
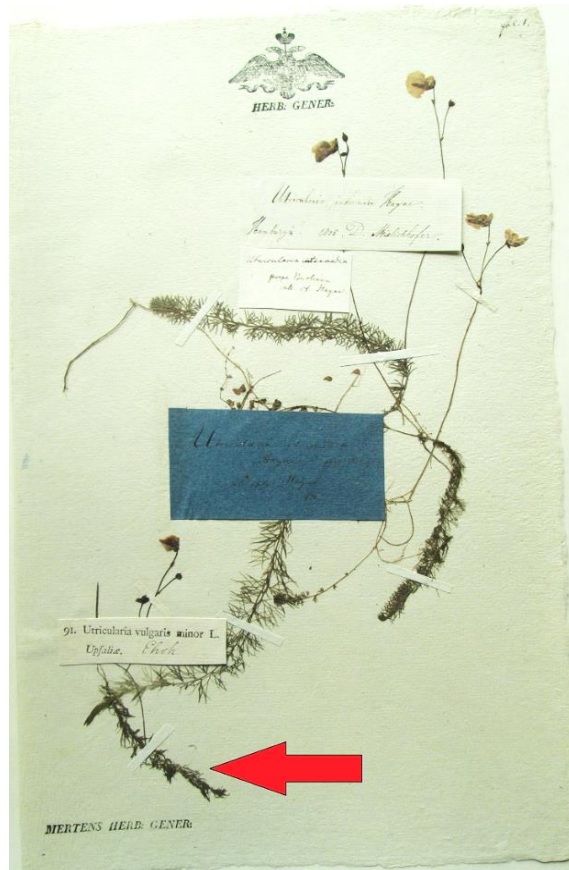
Figure 2. Herbarium sheet of *Utricularia intermedia* found in LE including a specimen (red arrow) eligible as lectotype.
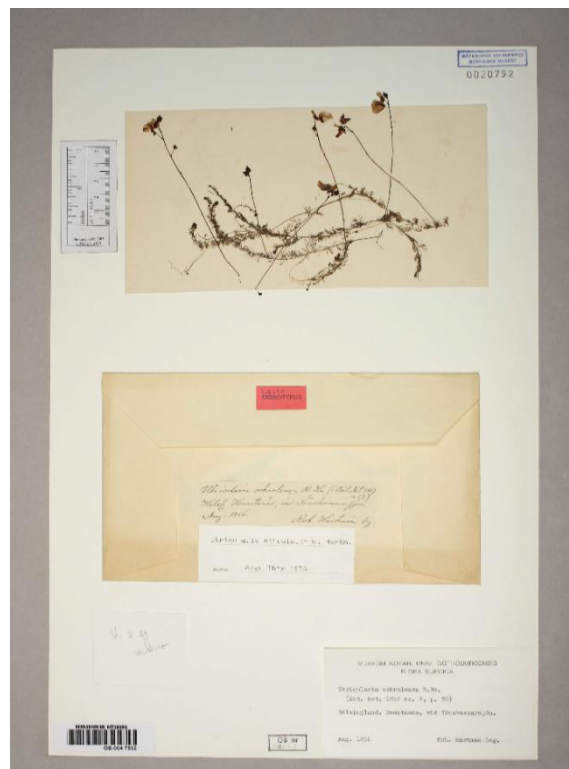

Figure 3. Lectotype of *Utricularia ochroleuca* conserved at GB.

Figure 4. Holotype of *Utricularia stygia* conserved at S.

Figure 5. Herbarium sheet of *Utricularia bremii* conserved in ZT including a specimen eligible as lectotype.
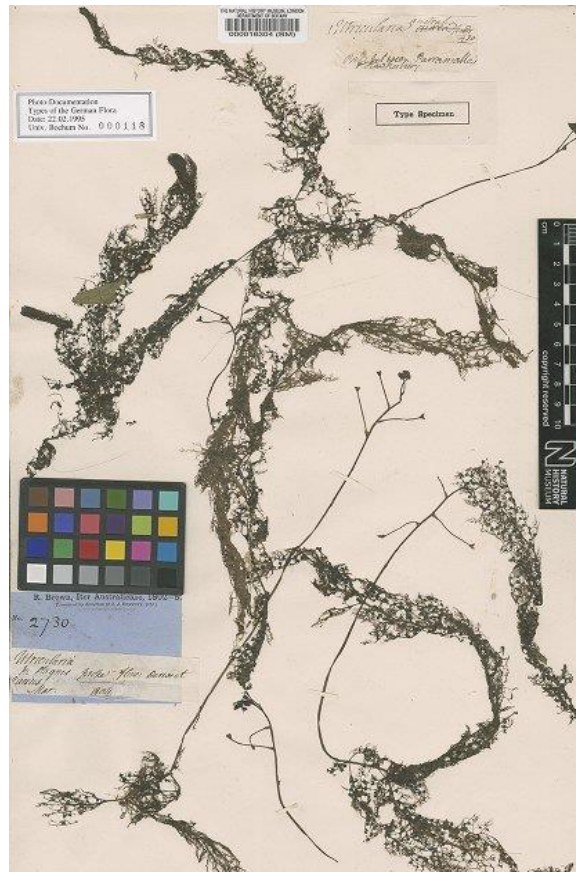
Figure 6. Lectotype of *Utricularia australis* conserved at BM.

Taxonomic and Systematic problems

Within the same aggregate, the species share an almost identical shoot morphology, so that distinguishing them is a very hard task if flowers are absent. However, some authors described features of the stolons that might help to discriminate units, even without flowers. Indeed, many populations of these species do not flower or rarely do, especially those populations belonging to mostly sterile species, such as *U. australis*, *U. bremii*, *U. ochroleuca* and *U. stygia*, but also *U. intermedia* (Taylor 1989). Thus, the best way to identify species would be looking at the discriminating features of vegetative parts, proposed by various authors during times. However, the reliability of these features has never been tested from a statistical point of view. Hereafter I refer to leaves as the branched parts departing perpendicularly from central axis of the shoot, not to traps, which are the true leaves of the plant.

According to available literature regarding the vegetative parts, it is possible to distinguish *U. australis* and *U. vulgaris* L. by the occurrence (or not) of the teeth (papillae), from which setulae arise, on the lateral margin of the ultimate leaf segments.

Also the rhizoids are reported to be different, short and with opposite bracts in *U. vulgaris*, much slender and with alternate bracts in *U. australis* (Thor 1988, Taylor 1989, Moeslund et al. 1990, Gariboldi & Beretta 2008). The two species are often considered not to co-occur (Meister 1900), but Taylor (1989) reported at least two (not specified) sites in England, characterized by acid bogs located at calcareous foothills, where the species grow together and also reported their co-occurrence in southwestern France. However, no more insight on these localities was given. In Italy, sometimes, these species have been reported together (e.g. Lago di Pratignano, Modena), but in this country they have been very often confused each other (Gariboldi & Berretta 2008). Generally, we can say that it is rare to find the two species together, also considering their (slight) differentiation in water conditions preference (see species descriptions chapter). Contrary to what usually reported, Gariboldi & Beretta (2008) stated that *U. australis* tolerates eutrophic waters better than the other *Utricularia* species, but without any further annotation.

Concerning *U. intermedia*, *U. ochroleuca* and *U. stygia*, the first can be discriminated from the other two species by the absence of traps on the green stolons, the number of teeth on the leaf margin and the apex shape of the ultimate leaf segment (Thor 1988, Taylor 1989, Tassara 2002, Adamec 2007b, Płachno & Adamec 2007). *Utricularia ochroleuca* and *U. stygia* can be distinguished by the angle between the short arms of the quadrifid glands and the shape of apical leaf segment (Thor 1988, Tassara 2002, Schlosser 2003, Płachno & Adamec 2007, Fleischmann & Schlauer 2014). However, probably the most used character for species discrimination is the angle included between the shorter arms of the quadrifid glands, details of which are discussed below.

The vegetative parts of *U. bremii* and *U. minor* are basically identical, even if the former one generally looks more robust and bears leaves divided up to 50 segments, while the latter up to 25 (Taylor 1989). In addition, in the recently published Flora Gallica (Tison & de Foucault 2014), it is reported that *U. bremii* bears quadrifid glands larger than *U. minor* (70-100 μm vs. 40-70 μm).

One of the most intriguing arguments is about the use of the quadrifid glands inside the traps as a diagnostic tool. Thor (1988) stated that all the Scandinavian species (6 out of 7 occurring in Europe, lacking *U. bremii*) might be distinguished by the features of these glands. Actually, Thor splitted *U. ochroleuca* in two species, *U. ochroleuca* and *U. stygia*, claiming that, besides the flowers, they have different angles between shorter arms (more obtuse in *U. ochroleuca*) and between shorter and longer arms (more obtuse in *U. stygia*).

Since its description, *U. stygia* has been recorded at many other sites throughout Europe (Schlosser 2003, Adamec 2007b, Płachno & Adamec 2007, Fleischmann & Schlauer 2014), thus showing a distribution wider than *U. ochroleuca*. However, other authors show some skepticism about the separation of the two species according to the keys proposed for Scandinavian populations (Adamec & Lev 2002, Schlosser 2003, Płachno & Adamec 2007). They criticize Thor for separating species only considering these populations, without studying those outside of Scandinavia. In their opinion, the globally low number of statistically investigated populations might have led to an underestimation of the variability of the features of quadrifid glands, as well as an overestimation of differences between the two putative species.

Concerning karyological aspects, for the central European species, Casper & Manitz (1975) conclude that the chromosome number is $2n = 44$, but various other numbers, varying from 36 to 48 were also recorded by them for some of these species, representing perhaps a series of dysploid vegetative apomicts (Taylor, 1989). To date, chromosome numbers have been reported for all European species, except for *U. stygia*, although some counts reported for *U. ochroleuca* could pertain actually to *U. stygia*.

Nevertheless, genome size has been estimated for all European species (Veleba et al. 2014), with two species, *U. bremii* and *U. stygia*, showing largest genomes (1C = 299 and 315 Mbp, respectively) compared to the others, which on the other hand show more or less the same size (1C ≈ 200 Mbp).

As concerns the phylogenetic relationships of European species, little information is available, that can be derived from the few existing molecular analyses, properly targeted to phylogenetic reconstructions at whole-genus level (Müller & Borsch, 2005). Several papers have focused with Lentibulariaceae, including some sequences of *Utricularia* (Jobson & Albert, 2002; Jobson et al., 2003; Müller et al., 2004, 2006). All the studies mentioned above have been performed exclusively using plastidial markers and including just three (*U. australis*, *U. intermedia* and *U. vulgaris*) out of the seven European species, each represented by a single sequence. From these analyses few consideration can be made concerning European species: 1) *U. australis* and *U. vulgaris* are more closely related respect to *U. intermedia*, as expected on morphological grounds, and 2) plastidial haplotypes of *U. australis* are closer to *U. vulgaris* than to putative parental species *U. macrorhiza*, partially consistent with the involvement od the latter species as male parental in the origin of *U. australis,* as hypothesised by Kameyama et al. (2005).

Rahman (2006, 2007) provided a different approach for analyzing phylogenetic relationships among aquatic species of *Utricularia*, by means of RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeat) markers and clustering (UPGMA and Neighbour Joining) for evaluation of overall similarity. In these analyses also some European species such as *U. australis*, *U. bremii*, *U. intermedia*, *U. minor* and *U. vulgaris* were included. In these studies, the close relationship between *U. australis* and *U. macrorhiza* was confirmed, as well as their similarity with *U. vulgaris*. Interestingly, *U. bremii* and *U. minor* failed to cluster together, as expected considering their extreme morphological affinities, but the latter resulted close to *U. gibba*, while the former with *U. intermedia*. However, the few species included in these studies do not allow inferring a complete phylogenetic hypothesis for European species. Moreover, the high molecular rates of evolution of *Utricularia* species and the putative massive presence of indels may considerably affect the phylogenetic results obtained with this DNA fingerprinting approach (Weising et al. 2005).

Objectives of the thesis

Since the high interest for carnivorous plants and particularly for the genus *Utricularia*, from evolutionary, eco-physiological, anatomical and many other points of view, and the relatively low taxonomic knowledge but high conservation interest of European species, a focus on the seven species occurring in Europe from a biosystematic point of view was devoted in this study.

The main goal was to check whether reliable tools for species identification exist, avoiding the use of flowers, in order to provide a framework useful for the correct delimitation of distribution range of each species, and to investigate the phylogenetic relationships occurring among taxa. Therefore, an investigation of selected morphological characters of the vegetative parts was performed using two different morphometric approaches: traditional and geometric.

In addition, an attempt to apply a DNA Barcoding approach was made on all these species, and contextually the same DNA sequences were used for phylogenetic reconstructions.

The use and utility of Geometric morphometrics

Geometric approach transforms a descriptive character, as the shape, in numbers (Cartesian coordinates), making possible the use of multivariate statistics (PCA, DA, etc.), preserving the original relationship among the points and treating all the shape variables with the same unit (Mitteroecker & Gunz 2009, Viscosi & Cardini 2011). For *shape*, in morphometrics, is meant any geometric property of an object that is independent by size, position and orientation of that given object, whereas the combination of shape properties and size is defined as *form* (Mitteroecker & Gunz 2009). Geometric morphometrics represents a very useful and relatively simple tool for describing shapes of many biological structures, since it minimizes the number of measurements, providing a more exhaustive view and facilitating the computations of values. The most used and best understood (mathematically and statistically) method for landmarks characterization is the Procrustes method (Rohlf & Slice 1990, Bookstein 1996, Small 1996, Dryden & Mardia 1998). *Landmarks* are loci characterized by Cartesian coordinates and a name (e.g. the point on the uppermost right, the point where the arms converge, etc.) that defines its position in the structure. All the chosen landmarks define the configuration of a structure. Each landmark of a given configuration has a corresponding landmark, with the same name (i.e. the same position), in all the other configurations (homology; Bookstein 1991). They are usually located by investigators in somehow easily detectable areas of the biological structure investigated, in order to minimize the arbitrariness of the choice for preserving as much as possible the assumption of homology.

Procrustes superimposition is a method to transform raw coordinates in shape coordinates, separating shape from size and other parameters as orientation and position on the space. This procedure is a least-square approach involving three main steps of computation (Mitteroecker & Gunz 2009): translation, scaling and rotation. In the translation step, objects are aligned according to their centroid, that is all the objects have the same centroid usually sent to the origin of the coordinates system. The second step involves the scaling of all landmarks configurations so that they all have the same size, expressed by the *Centroid size*. Centroid size is the square root of the summed squared deviations of each coordinate from its centroid. In the final step, all configurations reach the same orientation by means of rotations around the centroid. With only two configurations, one of the two, after being translated and scaled, is rotated around the centroid size until the sum of the squared Euclidean distances between the homologous

landmarks is minimal. For more than two configurations, an iterative algorithm called *Generalized Procrustes Analysis* (GPA) is applied (Gower 1975, Rohlf & Slice 1990). GPA involves the rotation of all configurations to one arbitrary configuration by means of the least square approach used with two configurations. Then, these new coordinates are averaged crating a consensus configuration and all configurations are rotating again to fit this consensus, producing again new coordinates. The new coordinates are averaged, representing the template for the next iteration.

GPA algorithm produces Procrustes coordinates and their consensus shape (obtained by averaging Procrustes coordinates) is the shape whose sum of squared distances to the other shapes is minimal. The individual differences from the consensus shape are called *Procrustes residuals* (Dryden & Mardia 1998).

The use of Procrustes coordinates in morphometrics results in a different statistical approach respect to traditional morphometrics, particularly concerning multivariate analysis. In traditional approach, multivariate analysis is applied to a different range of measurements, as distances, distance ratios, angles, counts, etc., which are often characterized by different units and ranges of variation. Thus, measurements are often log transformed and standardized to unit variance, resulting in analyses usually based on correlation matrices and in which different multivariate techniques and tests should be taken into account in order to investigate the entire set of data as best as one can. In geometric morphometrics, the shapes variables are all expressed in the same unit (Procrustes coordinates), so that analyses are based on covariance matrices and multivariate methods, such as *Principal Component Analysis* (PCA), correspond to actual shape or shape deformations of the original configurations, allowing the visualisation of the results (Mitteroecker & Gunz 2009).

The most famous and used method for the visualization of such deformations is the *Thin Plate-Spline* (TPS) method (Bookstein 1989, 1991). By this method is possible to compute *deformation grids*, which are pictures that represent differences between shape of different objects or changes of shape in the same object, an approach mainly due to D'Arcy Thompson (1915, 1917). TPS is a method based on the interpolation of the space between two configurations as being as smooth as possible (Gunz & Mitteroecker 2013). The best *smoothness* is reached minimizing the *bending energy* passing from a configuration to another, as the bending energy is defined by the integral of the squared second derivatives of the deformation (Mitteroecker & Gunz 2009). The bending energy

express the degree of localization of a deformation, where a high bending energy means a highly localized change of shape with dramatic change in nearby landmark position, while a low bending energy means a change over a large surface of the configuration with landmarks locally arranged more or less in the same way than before the deformation.

DNA Barcoding approach

Since its original description (Hebert et al. 2003), the barcoding approach has gained more and more credit among taxonomist as a powerful tool for species identification. Only in relatively recent years barcoding has been applied successfully also to plants (Hollingsworth et al. 2009, 2011).

Barcoding was first developed for species identification of animals using mitochondrial cytochrome *c* oxidase I gene (COI) sequences. The simple goal was to find peculiar DNA regions exclusively possessed by a species in a certain group of organisms. Similarly to mitochondrial markers for animals, plastidial markers have been proposed for applying barcoding approach to plants (Hollingsworth et al. 2009, 2011), but these markers revealed some problems. Indeed, being generally maternally inherited, plastidial markers are related to seed dispersal, but seed dispersal usually cover much shorter distance than pollen (Ghazoul 2005). Thereafter, plastidial genes may provide an underestimation of gene flow (Naciri et al. 2012), which instead, when conspicuous, represents a fundamental requirement for discriminating species (Petit & Excoffier 2009).

In addition, plants still represent a group of organisms showing peculiarities such as massive polyploidization, hybridization, introgression, clonal and/or unusual sexual reproduction, which provide constant difficulties from both the identification and phylogenetic reconstruction points of view. Also, identification of plants mainly relies on morphological and/or micromorphological traits, so that the concept of species is strongly related to morphospecies concepts more than to molecular concepts.

For these reasons, multilocus and multigenomic approaches are needed for making barcoding applicable to plants. The multilocus approach (Fazekas et al. 2008) deals with the possibility to use different plastidial markers combined, not only the standardized markers originally proposed for plant organisms, such as *rbc*L and *mat*K (Hollingsworth et al. 2009, 2011, Sandionigi et al. 2012). In this way, the discrimination between very closely related species and the avoiding of low variable markers (weak signal) can be easier. Multigenomic approach deals with the choice of markers belonging to both

plastidial and nuclear (mostly Internal Transcript Spacers, ITS marker) genomes (China Plant BOL Group 2011), so to ease the recognition of hybridization events and other misleading events such as introgression and incomplete lineage sorting after recent speciation, which can deeply affect species discrimination.

In the end, even when all these precautions have been taken to deal with barcoding approach, it could be not enough, because one of the most trouble-making issues could be the sampling. In fact, intraspecific variability as well as interspecific uniformity can be alternative and sometimes simultaneous troubles impeding DNA barcodes detection in several groups of plants. In order to diminish these troubles, more populations of each species are needed and, of each of them, more individuals as possible have to be collected (Zhang et al. 2010, Bergsten et al. 2012).

Once the sequences have been produced, for defining the barcode of a species, these sequences have to be included in a database, which should be as rich as possible. Unfortunately, the richest sequence database available is GenBank http://www.ncbi.nlm.nih.gov/genbank/, which contains many incorrect or out of date entries and, very often, for many species just one sequence is available. These errors may lead to an incorrect definition of the barcode to choose. On the other hand, Barcode of Life Data Systems (BOLD), the database set up by the CBOL, is still largely incomplete (Sandionigi et al. 2012). However, we can define a barcoding approach even in the case in which very closely related species, very difficult to discriminate by morphology, may be distinguished by differences in DNA sequences. For this purpose, it would be better to use the markers suggested by BOLD, i.e. *rbc*L and *mat*K, to respect the universality that should characterize the DNA Barcoding approach. Nevertheless, in several groups and particularly those consisting of very closely related species, these markers may be little or no informative and/or difficult to apply. Therefore, other markers can be used with the specific goal to discriminate species, despite they cannot be included in BOLD, since they do not respect the database guideline.

In the case of big datasets with a wide range of species and sequences, the choice of the data analysis method is not trivial and it could deeply affect the results. These methods belong to three categories: similarity methods, tree-based methods and character-based methods. Each of this method has advantages as well as flaws, depending on the management of data (Sandionigi et al. 2012), but character-based methods look very promising and their accuracy has been demonstrated in comparative studies on the

different methods (Van Velzen et al. 2012). The limitation of these character-based methods are due to computational or alignment problems, both typical of large datasets (Sandionigi et al. 2012). When few closely related species have to be discriminated, character-based methods could represent a successful tool. In case of low number of species to compare, a direct investigation by eye of the alignment can sometimes be accomplished, especially when the degree of variability of the chosen markers allows it.

MATERIAL & METHODS

General sampling

For all European species of *Utricularia,* fresh shoots were sampled, each species usually represented by two populations. In all European countries of occurrence (Germany, France, Poland, Czech Republic, Finland and Sweden), *U. ochroleuca* is subject to strict conservation programmes, then for this species only cultivated plants (Collection of aquatic and wetland plants, Institute of Botany, Třeboň, Czech Republic) were available, all originally collected from Třeboň basin area in the Czech Republic. For each population of the remaining species, five vegetative shoots were collected at least 30 m far from one another, according to the population area. For *U. ochroleuca*, only four fresh shoots were available, taken from ex-situ cultivated plants, but originally collected very distant from one another (more than 10 km). For teeth analysis of *U. ochroleuca*, ten herbarium specimens collected from two different sites (Świnoujście, Poland, and Lac de Longemer, France), five for each site, were also investigated. For leaf apex angle, also five shoots from herbarium specimens collected in Świnoujście, Poland, were investigated. In the molecular analyses, samples labelled with AD acronym were kindly provided by L. Adamec and all of them were originally collected in Třeboň Basin (South Bohemia, Czech Republic). See Table 1 and Appendix II for acronyms of populations investigated and further details on sampling.
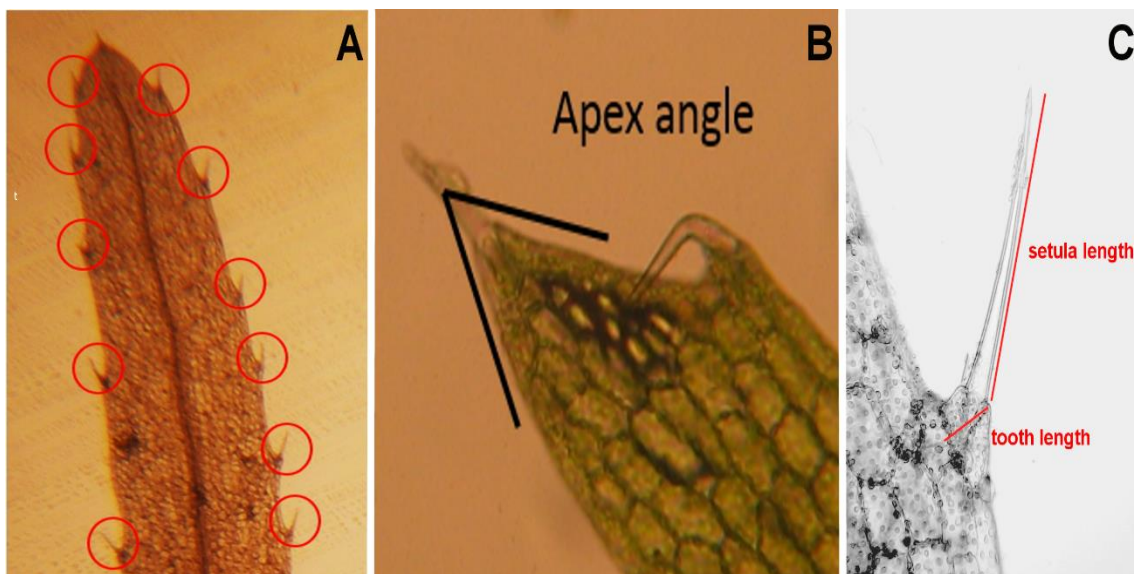
'Traditional' morphometric analysis

For species in *U. intermedia* aggr. teeth along the ultimate segment of the leaf margin and the angle at the apex of ultimate leaf segments were investigated (Figs. 7A and 7B). For species in *U. vulgaris* aggr. the ratio between the setula (trichome) length and the teeth (from which setulae arise) length was studied (Fig. 7C). I believe that even in *U. vulgaris*, contrarily to what sometimes reported in literature (Taylor 1989, Moeslund et al. 1990, Gariboldi & Beretta 2008), teeth along the leaf margin do exist, as already stated by other authors (Pignatti 1982, Thor 1988, Fleischmann & Schlauer 2014). However, in *U. vulgaris* it is common to find leaf margins with few and small teeth or no teeth at all, whereas in *U. australis* it is extremely rare to find margins with no teeth. For *U. minor* aggr. no morphological features with the traditional approach were investigated, because of the difficulties to find some suitable for statistical analysis.

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For the analysis of teeth and apex angles of ultimate leaf margin in *U. intermedia* aggr., three segments per leaf, two leaves per individual were measured. The three segments with more teeth were chosen for teeth analysis, while for apex angle, segments were randomly chosen. The tooth length has been measured from the most distant point of the tooth from the margin to the basis of the tooth, at a right angle to the margin. The length of the setula has been measured from the setula end to the setula base in contact with the tooth (see also Fig. 7C).

For the analysis of setula length/tooth length ratio in *U. vulgaris* aggr., one ratio per segment in three segments per leaf, two leaves per individuals, five individuals per population, in population and herbarium specimens, were estimated. For GEK, only two individuals were totally sampled. For herbarium specimens, three segments per leaf, two leaves per individual, one individual per sheet were considered: four sheets from different sites for *U. australis*, ten sheets from different sites for *U. vulgaris*.

For all these analyses, pictures were measured using ImageJ (Rasband 1997). Non-parametric tests such as Kruskal-Wallis for equal medians and Mann-Whitney pairwise analyses (Bonferroni correction applied) were performed for all morphometric analyses, at both species and population levels. Pearson's correlation test was performed to test the correlation between leaf apex angle and number of teeth on leaf margin in *U. intermedia* aggr. All these analyses were carried out with the software PAST 3.07 (Hammer et al. 2001, Hammer 2014).

Figure 7. Characters evaluated by traditional morphometrics. A) Number of teeth along leaf margin in *U. intermedia* aggr., B) angle at the apex of leaf segment in *U. intermedia* aggr. and C) length of the setula/length of the tooth in *U. vulgaris* aggr.

Table 1. Populations sampled for morphometric and molecular analyses. In brackets, number of shoots complexively used for molecular analyses.

Species	Population	Acronym	Number of shoots	Analysis
U. australis	Viareggio, Tuscany, Italy	ITV	5 (3)	Setula to tooth ratio, Glands GM, Molecular analysis
	Oranienbaum Heide, Saxony-Anhalt, Germany	GEO	5 (3)	Setula to tooth ratio, Glands GM, Molecular analysis
	Herbarium specimens from various localities*	HS	4	Setula to tooth ratio
U. bremii	Lake Monticolo, Trentino-Alto Adige, Italy	ITM	5 (7)	Glands GM, Molecular analysis
	Katzensee, Zurich, Switzerland	SWK	5 (3)	Glands GM, Molecular analysis
U. intermedia	Lake Bezymannoyoe, Leningrad Oblast, Russia	RUB	4 (4)	Glands GM, Molecular analysis
	Lake Michurinskoye, Leningrad Oblast, Russia	RUM	5	Number of teeth, Leaf apex angle, Glands GM, Molecular analysis
	Giwiggsenriet, Zurich, Switzerland	SWG	5 (3)	Number of teeth, Leaf apex angle
U. minor	Lake Bezymannoye, Leningrad Oblast, Russia	RUB	5 (3)	Glands GM, Molecular analysis
	Lake Michurinskoye, Leningrad Oblast, Russia	RUM	5	Glands GM
	Passo del Tonale, Trentino Alto-Adige, Italy	ITT	5 (5)	Molecular analysis
U. ochroleuca	Třeboň Basin, Czech Republic	CZ	4 (3)	Number of teeth, Leaf apex angle, Glands GM, Molecular analysis
	Lac de Longemer, Vosges, France*	FR	5	Number of teeth
	Świnojuście, Poland*	PL	5	Number of teeth, Leaf apex angle
U. stygia	Lake Monticolo, Trentino-Alto Adige, Italy	ITM	5 (7)	Number of teeth, Leaf apex angle, Glands GM, Molecular analysis
	Ambitzgi, Zurich, Switzerland	SWA	5 (3)	Number of teeth, Leaf apex angle, Glands GM, Molecular analysis
U. vulgaris	Lake Bezymannoye, Leningrad Oblast, Russia	RUB	5 (3)	Setula to tooth ratio, Glands GM, Molecular analysis
	Lake Michurinskoye, Leningrad Oblast, Russia	RUM	5 (3)	Setula to tooth ratio, Glands GM, Molecular analysis
	Klieken, Saxony-Anhalt, Germany	GEK	2	Setula to tooth ratio
	Herbarium specimens from various localities	HS	10	Setula to tooth ratio

Geometric morphometric analysis

From each individual, five fully developed traps were randomly taken and analysed under light microscope. The traps were carefully halved in order to investigate the glands. For each trap, five quadrifid glands were photographed, avoiding those occurring close to the mouth, where many abnormally shaped glands occur along with two-armed (bifid) ones (personal observations). In the selected trap's area, glands were randomly chosen. TPS files (Rohlf 2010) were created using TPSUtil software, applied to glands' pictures. The TPS file was processed with TPSDig for landmarks positioning. Five landmarks were

chosen, four corresponding to the tips of the arms and one located on the centre of the gland, where all arms converge (Fig. 8; see also Thor 1988). The digitized TPS file was converted in NTS file and imported in MorphoJ software (Klingenberg 2011), where the specimens were aligned using the function New Procrustes Fit, that creates new coordinates (i.e. Procrustes coordinates). After fitting specimens, Procrustes coordinates were imported in PAST 3.07 software (Hammer et al. 2001, Hammer 2015) and a PCA (Principal Component Analysis) was performed on the Procrustes coordinates. Tests such as Mardia's tests for skewness and kurtosis were applied along with an omnibus Doornik and Hansen test (Mardia 1970, Doornik & Hansen 1994). Besides the shape of the glands, also their centroid size was calculated in order to evaluate differences between and within aggregates. Centroid size was calculated by means of TPSrelw and then imported in PAST (see previous chapter) for univariate statistical analysis (Kruskal-Wallis test). These analyses were first performed on the complete set of species, and then on species within the same aggr. For testing variation in traps and individuals, a function called Procrustes ANOVA, available with MorphoJ, was used. According to Viscosi & Cardini (2011), in order to avoid misspecification of factors affecting the computation of F in Procrustes ANOVA, I manually computed the F ratio using 'gland' as random effect and 'trap' as main effect and using 'trap' as random effect and 'individual' as main effect. To calculate F I divided the mean sum of squares (MS) of the highest hierarchical rank (i.e. trap in the first case, individual in the second case) by the mean sum of squares of the lowest hierarchical rank (i.e. gland in the first case, trap in the second case). For testing the significance of the F statistics, I used an F distribution calculator (http://davidmlane.com/hyperstat/F_table.html accessed 14/04/15) to obtain a P value. Procrustes ANOVA represents a preliminary analysis useful for evaluating the suitability of using mean values (mean shape of glands within a trap, mean shape of glands within an individual, obtained pooling mean shape of glands of each trap together). For both trap and individual levels, I pooled glands together using the "Average observation by" function available with MorphoJ. The datasets with averaged observations were imported in PAST.
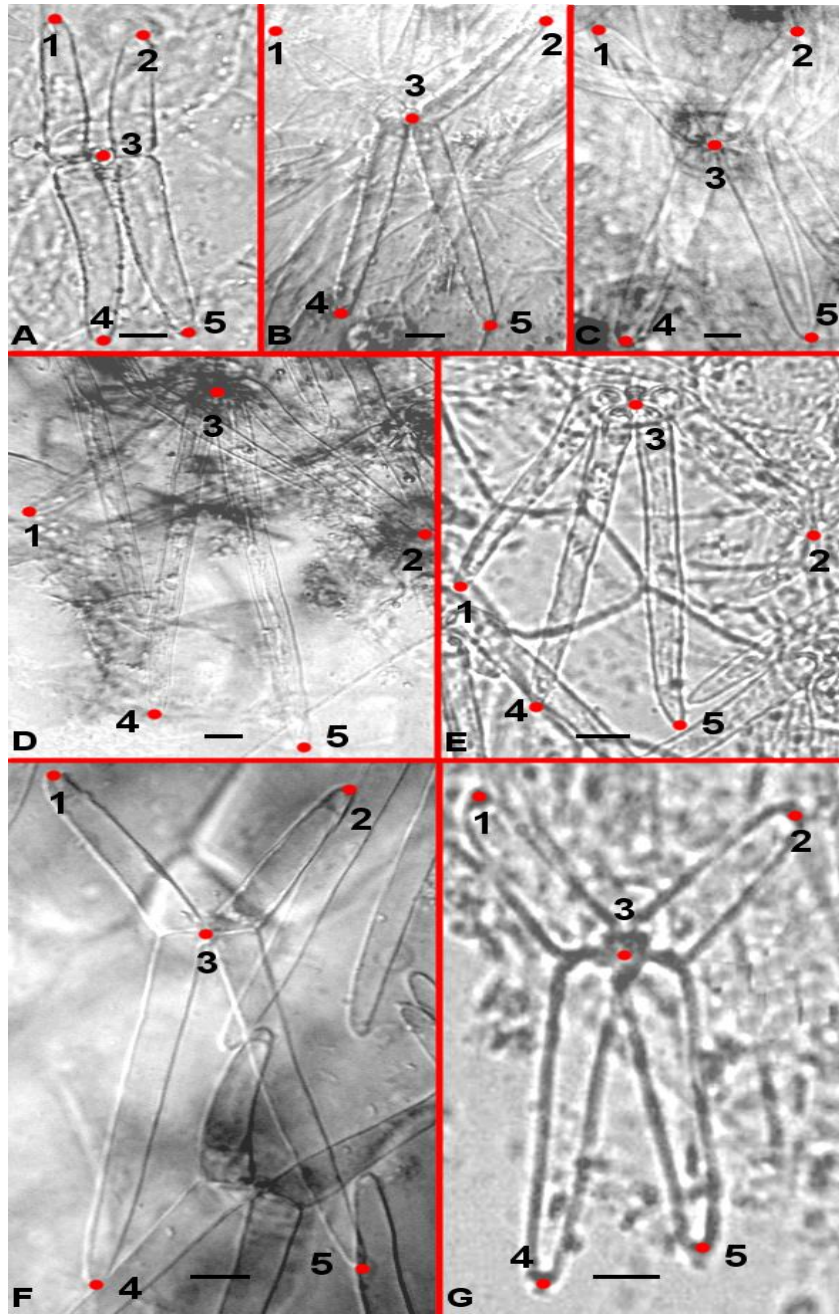
Figure 8. Quadrifid glands in European species of *Utricularia*. A) *U. intermedia*, B) *U. ochroleuca*, C) *U. stygia*, D) *U. bremii*, E) *U. minor*, F) *U. australis* and G) *U. vulgaris*. Micometric bar: 10 μm.

Then, I performed PCA on the new averaged dataset for comparing species and Discriminant Analysis (DA hereafter) for evaluating the correct species' attribution of traps and individuals as well as for testing groups differences (species and populations). Along with DA, also permutation tests were performed for testing group differences. Covariation between size and shape was also evaluated to test allometry effect (Viscosi & Cardini 2011). For this reason, a regression analysis of Procrustes coordinates as

dependent variable onto centroid size as independent variable was performed on observations averaged by trap. Permutation test with 10000 runs was performed for significance evaluation. The regression was calculated separately for each population investigated and when allometry resulted significant, size-correction was performed. Size-correction was obtained computing residuals from the regression of size onto shape. These residuals were used for further analysis such as PCA and DA, instead of Procrustes coordinates. For details on methodology for evaluating allometry, refer to Viscosi & Cardini (2011).

Molecular analysis

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 preserved in silica gel. DNA extraction was performed in some cases, following the protocol of Lodhi et al. (1994, modified), and in most of the cases using Plant II DNA extraction kit (Machery-Nagel). In both cases, plant tissues were first macerated in a mortar with liquid nitrogen.

Three markers, the plastidial *trn*L-*trn*F intergenic spacer and *rps*16 intron and the nuclear ITS (Internal Transcript Spacer) region (ITS1 + 5.8S + ITS2) were used for the analyses. Amplification of the three markers was performed using 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.

Sequences obtained were first aligned with Clustal X 2.1 (Larkin et al. 2007) and then manually corrected. Phylogenetic networks were built using SplitsTree 4 software, particularly the function Neighbour-Net (Huson & Bryant 2006). For details on splits and networks, see below in M&M. For phylogenetic trees, maximum parsimony and neighbour joining analyses were performed using PAUP 4.08b10 software (Swofford 2003).

Table 2. Primers and PCR conditions.

Conditions	ITS (White et al. 1990)			trnL-trnF IGS (Taberlet at al. 1997)			rps16 intron (Oxelman et al. 1997)		
	Temperature	Time		Temperature	Time		Temperature	Time	
Initialization	95 °C	1'		94 °C	1'30''		94 °C	1'30''	
Denaturation	92 °C	30''	×34	94 °C	1'	×35	94 °C	30''	×30
Annealing	50 °C	50''		52 °C	1'		56 °C	30''	
Elongation	70 °C	1'		72 °C	2'		72 °C	1'	
Final elongation	70 °C	10'		72 °C	15'		72 °C	15'	

DNA Barcoding approach

The guideline of Plant Working group of CBOL (http://www.barcoding.si.edu/plant_working_group.html) suggests the use of two plastidial markers, *rbc*L and *mat*K genes. Unfortunately, both these markers present some critical issues concerning *Utricularia*. The *rbc*L when used by other investigators, in a wide sampling approach of *Utricularia* species, revealed to be not suitable for Barcoding, because highly conservative (V. Fernandes Oliveira de Miranda amd S. Rodrigues da Silva, personal communication), thus potentially not effective on very close related taxa such as European species. On the other hand, according to Müller et al. (2004), *mat*K revealed to be extremely variable in *Utricularia*, showing the highest substitution rates found so far among angiosperms. Even if this peculiarity may be potentially useful for assessing species phylogenetic relationships, it would be somehow problematic from a barcoding point of view. Indeed, the high intraspecific variability could hide the signal for species discrimination. Moreover, this high variability determines difficulties in sequences alignment, with potential introduction of biases by the investigator who manually correct the alignment (e.g., indels length). Finally, primers for *mat*K are not so easily designable and detectable, making difficult the amplification and the subsequent production of reliable sequences (Hollingsworth et al. 2011).

For these reasons, I discarded these plastidial markers, despite strongly 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. These latter markers worked well in the study of Jobson & Albert (2002), even if their study was exclusively focused on phylogenetic reconstruction of a wide range of *Utricularia* species, not on the identification matter of a small group of closely related species. In addition, the availability of primers in literature and the relative easiness to obtain sequences, led me to choose these plastidial markers for my studies.

Considering the putative hybrid origin of some of the species included in my study and the fact that nuclear markers were never used in *Utricularia*, I decided to include ITS in my investigations. Anyway, ITS is an obvious choice to use as supplementary marker for Barcoding issue, as already proposed by other authors involved in CBOL Plant Working Group (China Plant BOL Group 2011, Hollingsworth et al. 2011, Li et al. 2011).

Splits and phylogenetic networks

Usually, evolutionary relationships between taxa are represented by phylogenetic trees, including those based on DNA sequences. The concept of phylogeny is based on the recognition of two phenomena leading the evolution: mutations and speciations. Instead, we know how evolution of organisms is also deeply affected by hybridization, introgression and horizontal gene transfer. Moreover, reconstruction of relationships between taxa are based on reconstruction of gene (or non-coding DNA regions) trees, which may not correspond to actual species trees, because of misleading events such as gene loss and duplication, incomplete lineage sorting or recombination. For these reasons, a single phylogenetic tree may not be an appropriate representation of different incompatible phylogenetic signals, whereas a phylogenetic network may be more suitable for this purpose (Fitch 1997). One way to represent conflicting signals in a phylogenetic network is the split graph.

Considering an unrooted tree *T*, a split is a partition of the taxa in two disjoint, non-empty groups or subtrees. In Fig. 9A is illustrated the number of splits occurring on the branches along the path from taxon *x* to taxon *y*. A collection of splits given by all the branches of *T* represents all the phylogenetic information related to this phylogeny and it is called a *set of splits* of *T*. A collection of splits is *compatible* when it is contained within the set of splits of some phylogenetic tree, otherwise is *incompatible*. Any compatible set of

splits can be represented by a phylogenetic tree, whereas an incompatible set of splits can be represented by a splits network. A *split network* is a more generalized type of phylogenetic graph that can represent any collection of splits, whether incompatible or not. For a compatible set of splits, it is always possible to represent each split with a single branch, and thus the resulting graph is a tree. In general, however, this is not possible and, in a split network, usually a band of *parallel branches* is required to represent a single split. In Fig. 9B is illustrated an example of incompatible splits and their graphic representation. Incompatible splits can be produced by any conflicting signal, deriving for instance by different trees produced by different genes of the same taxa, or even deriving by different columns of a single alignment. Hence, split networks somehow build up consensus graphs for phylogenetic reconstructions with conflicting phylogenetic signals. In a phylogenetic tree, the terminal branchs represent taxa and the internal nodes represent speciation events. Such a clear interpretation is not possible in a split network, which must be viewed more abstractly as a graph giving a visual representation of incompatible signals, showing how *treelike* a phylogeny, or certain parts of it, is. For theoretical background of splits and split networks, see Bryant & Moulton (2004), Huson (2005) and Huson & Bryant (2006).

One of the most used algorithms for constructing split networks is *Neighbour-Net* (Bryant & Moulton 2004). This algorithm is based on the distance method *Neighbour Joining* (NJ), used for phylogenetic trees (Saitou & Nei 1987). As in NJ an agglomerative process take place, starting from one node for each taxon and replacing each pair of nodes produced at each iterative step with a new composite node. Differently from NJ, in Neighbour-Net a pair of nodes is not combined and replaced immediately but only when a node is paired up a second time. At this point the three linked nodes are replaced by two linked nodes and the matrix is reduced and if there is still a node linked to two others a second agglomeration and reduction is performed. Then, the iteration process continues and finally a collection of splits is produced. For an intuitive explanation, see Fig. 9C.

Phylogenetic trees

As outgroup, sequences of *U. biloba* (*rps*16: GenBank voucher AF482561; *trn*L-F: GenBank voucher AF482634) and *U. resupinata* (*rps*16: GenBank voucher AF488527; *trn*L-F: GenBank voucher AF488533), available on NCBI, were chosen for plastidial phylogenies, based on the phylogenetic trees produced by Jobson et al. (2003). Plastidial

sequences of *U. gibba* (*rps*16: GenBank voucher AF482572; *trn*L-F: GenBank voucher AF482648) available on NCBI, were included in the dataset of phylogenetic tree, because its relationship with European species, particularly with *U. bremii*, found by Rahman (2006, 2007). Plastidial sequences of *U. macrorhiza* (*rps*16: GenBank voucher AF482581; *trn*L-F: GenBank voucher AF482657), available on NCBI, were included in phylogenetic tree because this species is a putative parental species of *U. australis*. Unfortunately no ITS sequence of *U. macrorhiza* was available on NCBI. For ITS phylogenetic tree, a sequence of *U. reniformis* (GenBank voucher DQ225108) from NCBI was tested as outgroup, but unsuccessfully. Indeed, this sequence revealed extremely variable in terms of substitutions and indels respect to ingroup sequences. ITS tree obtained using this outgroup was deeply unresolved and affected by long branch attraction error (Felsestein 1978).

Gaps were treated both as missing and as new state in all the phylogenetic trees computed. MP analysis was performed via heuristic search, with character considered unordered and all with equal weight. Sequences were randomly added. Starting trees were obtained via stepwise addiction and branch-swapping algorithm via TBR (tree-bisection-reconnection). Bootstrap with 10000 pseudoreplicates on both MP and NJ analyses was performed.

A
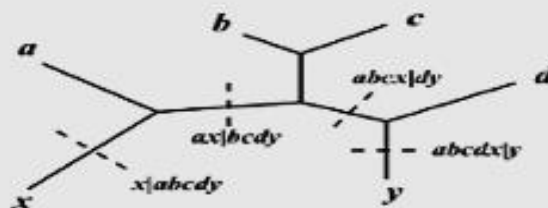
The splits graph for a set of compatible splits (i.e., a tree).
The splits for the branches along the path from *x* to *y* are given. These are
exactly the splits of the tree with *x* and *y* in different groups.

from Huson 2005

Consider the following two trees T_1 and T_2, for which the splits $S_p = \frac{\{a,b,c\}}{\{d,e\}} \in \Sigma(T_1)$ and $S_q = \frac{\{a,b,d\}}{\{c,e\}} \in \Sigma(T_2)$ are incompatible:

T_1 T_2 $SN(\Sigma)$
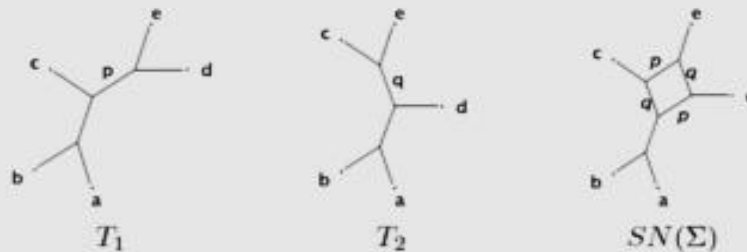
The "splits network" $SN(\Sigma)$ represents the incompatible set of splits $\Sigma :=$ $\Sigma(T_1) \cup \Sigma(T_2)$, using "bands of parallel edges" to represent splits that are incompatible with others

from Bryant & Moulton 2004

B
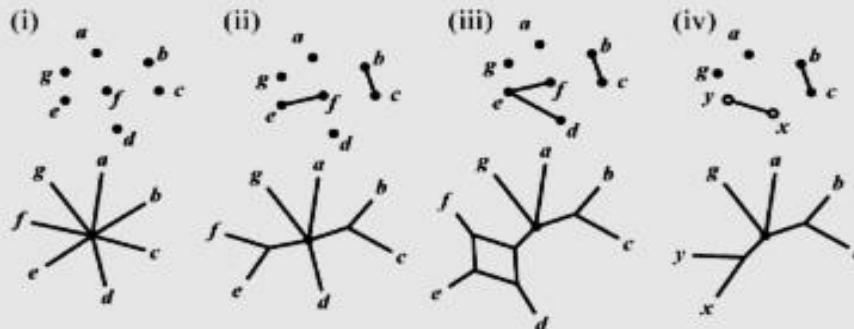
The agglomerative process for Neighbor-Net. (i) We begin
with each node representing a single taxon. (ii) Using the selection
criterion, we identify *b* and *c* as neighbors, as well as *e* and *f*. Unlike NJ,
we do not amalgamate immediately. (iii) We have identified *e* as
a neighbor of *d* (as well as *f*). Notice how the splits *ef|abcdg* and
de|acdfg are both represented in the splits graph. (iv) As *e* has two
neighbors, we perform a reduction, replacing *d*, *e*, *f* by *x*, *y*.

from Huson 2005

**Figure 9.** Illustration of A) Splits and B) Neighbour-Net analysis.

RESULTS

'Traditional' morphometric analysis

Comparisons of the number of teeth on leaf margin between species and between populations within *U. intermedia* aggr. are reported in Table 3A and in Fig. 10. P values (< 0.01) show that significant differences between sample medians at both species and population levels were found. Pairwise analysis shows that differences between species are all significant ($p < 0.01$), while, at population level, no differences were found between the two populations of *U. intermedia* as well as between the two populations of *U. ochroleuca* investigated on herbarium specimens (*U. ochroleuca* FR and PL in Table 3A). On the contrary, a significant difference was found between populations of different species, between the two populations of *U. stygia* ($p = 0.0085$), and between the population of cultivated fresh plants of *U. ochroleuca* and the two populations constituted by herbarium specimens. The boxplots (Figs. 10A and 10B) show that a certain distinction can be found between species as concerns teeth's feature, even if a slight overlapping of values exists.
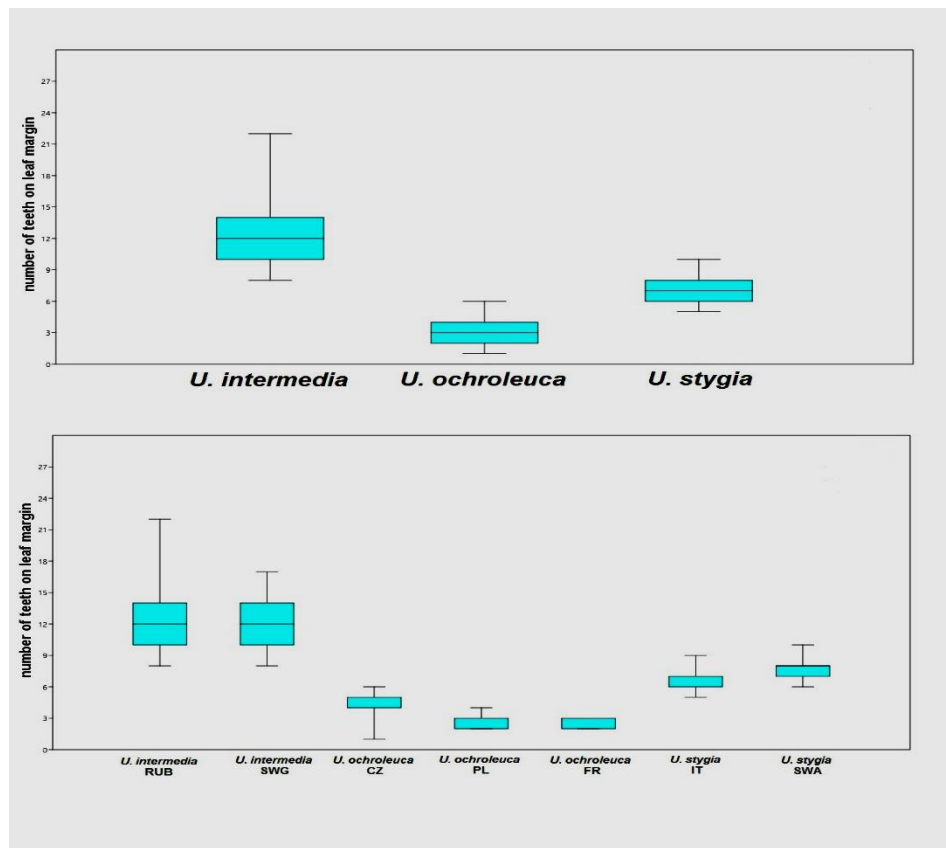
Figure 10. Boxplot: number of teeth along leaf margin in *U. intermedia* aggr. Species (above) and single populations (below)

Comparisons of apex angles on ultimate leaf segments between species and between populations within *U. intermedia* aggr. are reported in Table 3B and in Fig. 11. P values (< 0.01) show that significant differences between sample medians at both species and population levels were found. Pairwise analysis shows that differences between species are all significant (p < 0.01), while, at population level, no differences were found between the two populations of *U. intermedia* as well as between *U. intermedia* SWG and *U. stygia* IT (p = 0.1143) and between *U. ochroleuca* CZ and *U. stygia* SWA. This demonstrates that sometimes differences within species appear to be higher than between species in *U. intermedia* aggr. concerning leaf apex angle's feature. The population boxplot confirms that species are not clearly distinct and a considerable overlapping of values is found when comparing different species (Figs. 11A and 11B).
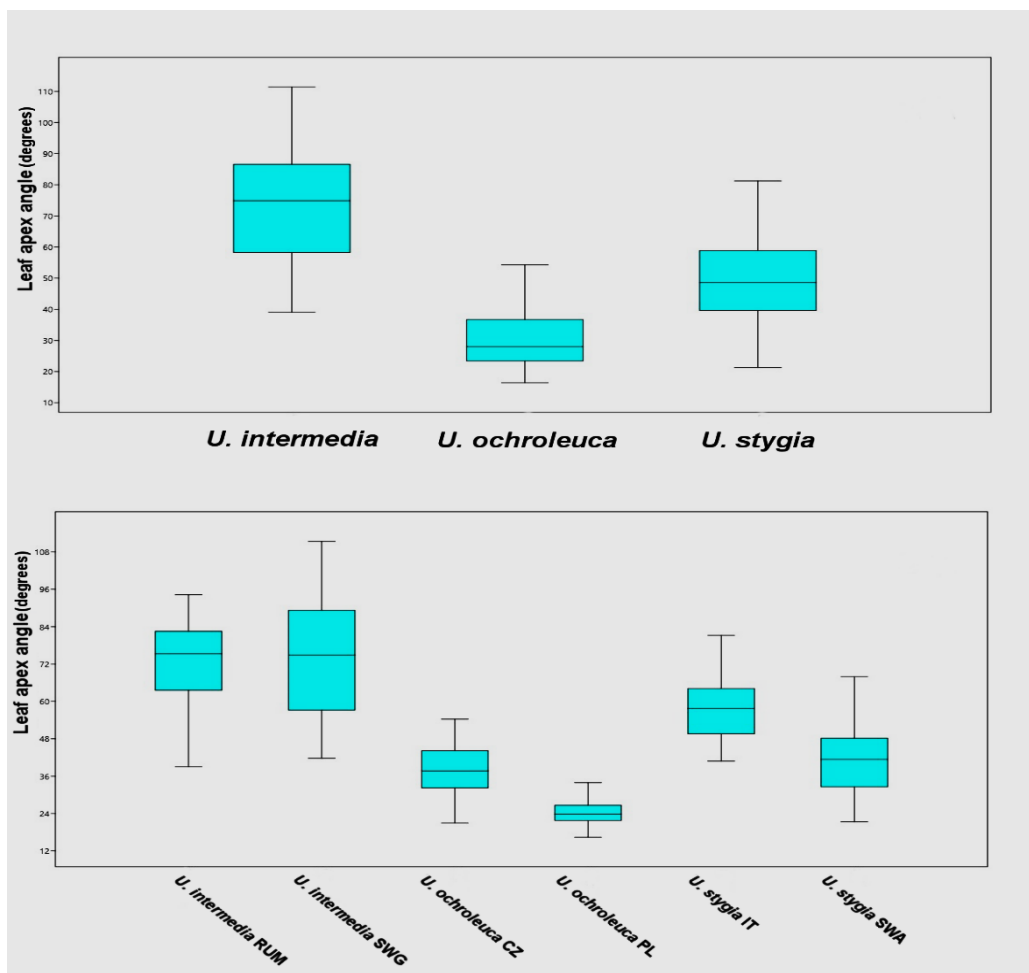
Figure 11. Boxplot: Leaf apex angle on ultimate segments in *U. intermedia* aggr. Species (above) and single populations (below).
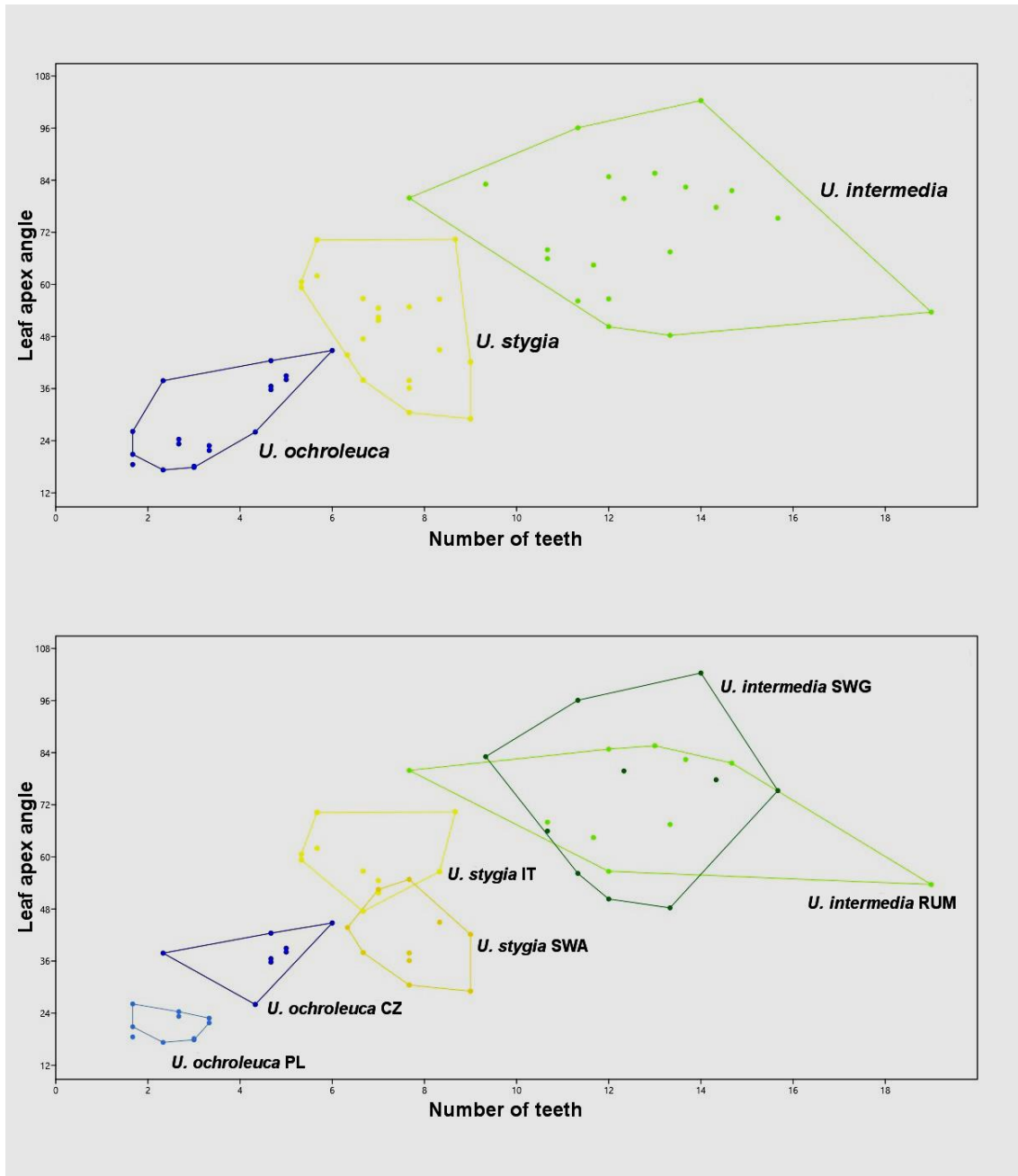
If the two features, the teeth on the leaf margin and the leaf apex angle, are combined in the same analysis, a certain separation between the three species can be found, with *U.*

intermedia generally showing the highest values for both characters, *U. ochroleuca* the lowest ones (Fig. 12A). However, also a distinction between populations within the same species in *U. ochroleuca* and *U. stygia* was found, whereas the two populations within *U. intermedia* neatly overlap (Fig. 12B). The two features investigated in *U. intermedia* aggr. are correlated using a Pearson test (r = 0.7746, p < 0.01). Generally, leaf segments with obtuse apex angle show higher number of teeth on their margin.

Table 3. 'Traditional' morphometric analysis. Mann-Whitney pairwise (Bonferroni correction) test. S = significant at 0.01 level, NS = not significant.

A) Number of teeth *U. intermedia* aggr. Mann-Whitney pairwise (Bonferroni correction)

	population	*U. intermedia*		*U. ochroleuca*			*U. stygia*	
		RUM	SWG	CZ	FR	PL	IT	SWA
U. intermedia	RUM	-	NS	S	S	S	S	S
	SWG	-	-	S	S	S	S	S
U. ochroleuca	CZ	-	-	-	S	S	S	S
	FR	-	-	-	-	NS	S	S
	PL	-	-	-	-	-	S	S
U. stygia	IT	-	-	-	-	-	-	S
	SWA	-	-	-	-	-	-	-

B) Leaf apex angle *U. intermedia* aggr. Mann-Whitney pairwise (Bonferroni correction)

	population	*U. intermedia*		*U. ochroleuca*		*U. stygia*	
		RUM	SWG	CZ	PL	IT	SWA
U. intermedia	RUM	-	NS	S	S	S	S
	SWG	-	-	S	S	NS	S
U. ochroleuca	CZ	-	-	-	S	S	NS
	PL	-	-	-	-	S	S
U. stygia	IT	-	-	-	-	-	S
	SWA	-	-	-	-	-	-

C) Setula/tooth length *U. vulgaris* aggr. Mann-Whitney pairwise (Bonferroni correction)

	population	*U. australis*			*U. vulgaris*			
		IT	GEO	HS	RUB	RUM	GEK	HS
U. australis	IT	-	NS	NS	S	NS	S	S
	GEO	-	-	NS	S	S	S	S
	HS	-	-	-	S	NS	S	S
U. vulgaris	RUB	-	-	-	-	S	NS	NS
	RUM	-	-	-	-	-	S	S
	GEK	-	-	-	-	-	-	NS
	HS	-	-	-	-	-	-	-

Figure 12. Combined analysis with number of teeth along margin leaf and leaf apex angle in *U. intermedia* aggr. Scatter plot of species (above) and of single populations (below).

The ratio of setula length to tooth length in *U. vulgaris* aggr. revealed significantly different medians according to species (p < 0.01). Considering pairwise analysis at population level, no differences were found between the populations of *U. australis*, while as concerns *U. vulgaris*, the population from Michurinskoye Lake shows no difference if compared to populations of *U. australis* and significant difference if compared with co-specific populations. The other three populations of *U. vulgaris* show no differences if compared one to each other (Table 3C). See also Fig. 13.
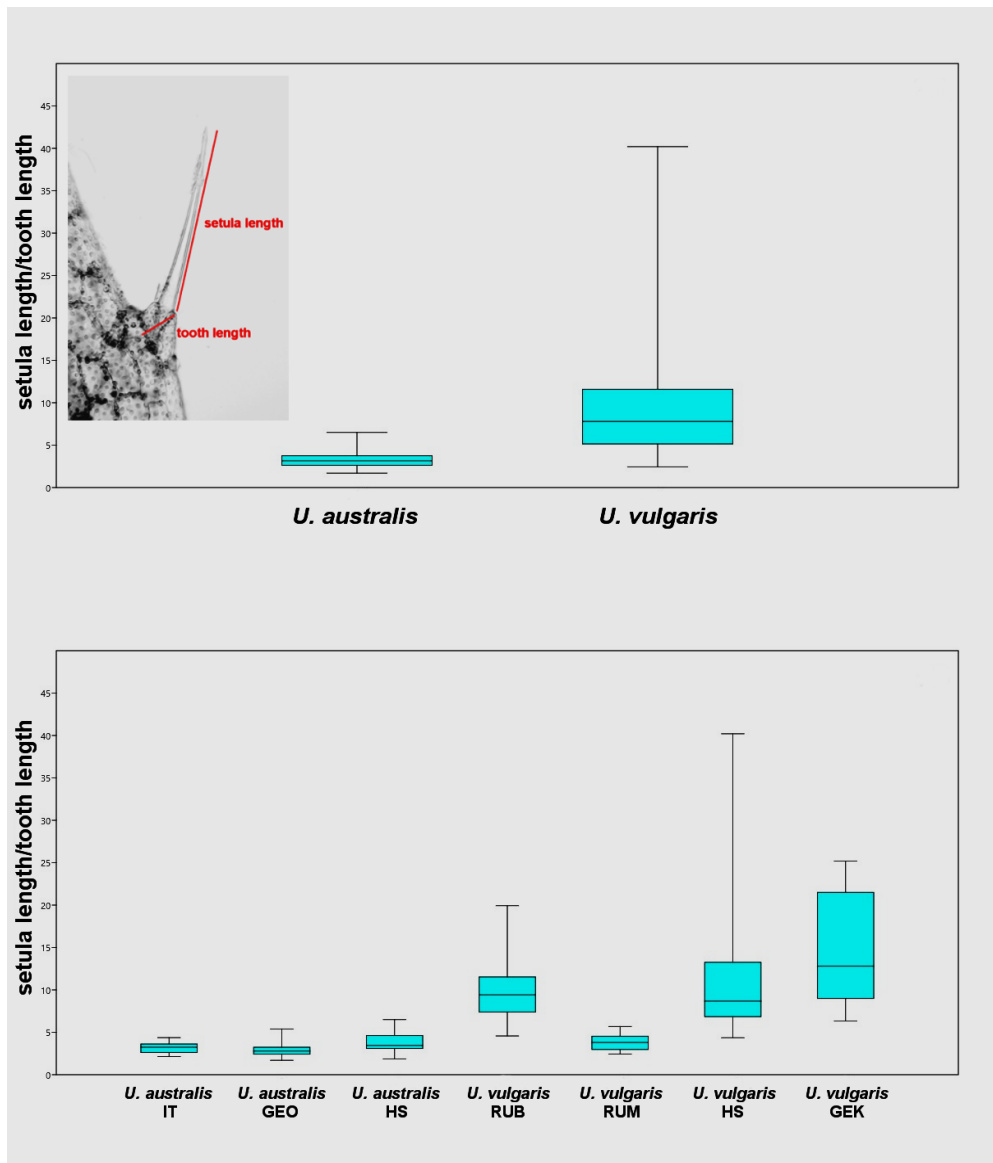
Figure 13. Boxplot of setula length/tooth length ratio in *U. vulgaris* aggr. A) Species and B) single populations.

Geometric morphometric analysis

Shape

All species

Multivariate normality tests revealed that data distribution (Procrustes coordinates) was significantly non-normal. Thus, for assessing differences between groups, a non-parametric test such as Permutation test was applied.

PCA performed on all European species was able to separate taxa belonging to *U. minor* aggr. from the rest of the species, but it was not able to discriminate neither species within

this aggregate nor species within and between other aggregates. The variance explained by the first axis is high (ca. 72%), while it is quite low for the rest of the axes (Figs. 14A, 14B, and 14C). Jackknifed confusion matrix calculated with the DA gave a percentage of correct classification around 53.5%. Permutation test (10000 runs) performed pairwise resulted significant for all pairs of species ($T^2 < 0.01$).

Procrustes ANOVA resulted to be significant in both cases evaluated (with individual and traps as random effect), indicating that differences between individuals are larger than differences within individuals and differences between traps are larger than differences within traps. Thus, analyses with averaged measurements of both traps and individuals were performed (Table 4). PCAs of measurements averaged both by traps and individuals show a separation of species better than using the whole glands dataset, especially concerning *U. bremii*, *U. intermedia*, *U. minor* and *U. stygia.* DA gave a jackknifed percentage of correct classification, 59.7%, for averaged traps analysis higher than using the whole glands dataset DA analysis, while lower, 52.4%, for averaged individuals (see also Table 5). T^2 test resulted significant for all species' pairs when measurements were averaged by traps, while, when measurements were averaged by individuals, it resulted non-significant for the following species pairs: *U. australis*/*U. vulgaris* ($T^2 = 0.067$), *U. bremii*/*U. minor* ($T^2 = 0.033$), *U. ochroleuca*/*U. stygia* ($T^2 = 0.0576$), and *U. ochroleuca*/*U. vulgaris* ($T^2 = 0.4619$).

Table 4. Procrustes ANOVA applied to all-species set and aggregates.

	MS gland	df	MS trap	df	MS individual	df	F = MS trap/MS gland	P	F = MS individual/MS trap	P
All species	0.01616	9444	0.06546	1884	0.30712	372	4.0508	0	4.6917	0
U. intermedia **aggr.**	0.00611	3444	0.01852	684	0.07717	132	3.0286	0	4.1668	0
U. minor **aggr.**	0.00919	2994	0.01639	594	0.03534	114	1.7830	0	2.1566	0
U. vulgaris **aggr.**	0.00393	2994	0.00781	594	0.02100	114	1.9873	0	2.6886	0

Table 5. Discriminant Analysis (allocation) and permutation test (significance reported in bracket, S = significant at 0.01 level, NS = not significant) on Procrustes coordinates in all-species set.

A) Discriminant Analysis all glands

	U. australis	*U. bremii*	*U. intermedia*	*U. minor*	*U. ochroleuca*	*U. stygia*	*U. vulgaris*	Total
U. australis	87	2 (S)	9 (S)	1 (S)	34 (S)	38 (S)	79 (S)	250
U. bremii	0 (S)	139	3 (S)	103 (S)	3 (S)	1 (S)	1 (S)	250
U. intermedia	0 (S)	0 (S)	183	0 (S)	2 (S)	40 (S)	0 (S)	225
U. minor	1 (S)	93 (S)	3 (S)	139	4 (S)	4 (S)	6 (S)	250
U. ochroleuca	6 (S)	0 (S)	9 (S)	2 (S)	38	25 (S)	20 (S)	100
U. stygia	11 (S)	1 (S)	45 (S)	2 (S)	45 (S)	142	4 (S)	250
U. vulgaris	53 (S)	2 (S)	15 (S)	3 (S)	40 (S)	25 (S)	113	250
Total	161	236	268	254	170	260	226	1575

Jackknifed correct attribution = 53.46%

B) Discriminant Analysis averaged by trap

	U. australis	*U. bremii*	*U. intermedia*	*U. minor*	*U. ochroleuca*	*U. stygia*	*U. vulgaris*	Total
U. australis	27	1 (S)	1 (S)	0 (S)	2 (S)	5 (S)	14 (S)	50
U. bremii	0 (S)	25	1 (S)	24 (S)	0 (S)	0 (S)	0 (S)	50
U. intermedia	1 (S)	2 (S)	37	0 (S)	0 (S)	4 (S)	1 (S)	45
U. minor	1 (S)	18 (S)	0 (S)	29	0 (S)	2 (S)	0 (S)	50
U. ochroleuca	3 (S)	0 (S)	2 (S)	0 (S)	7	3 (S)	5 (S)	20
U. stygia	3 (S)	1 (S)	2 (S)	1 (S)	3 (S)	39	1 (S)	50
U. vulgaris	8 (S)	2 (S)	2 (S)	1 (S)	9 (S)	4 (S)	24	50
Total	43	49	45	55	21	57	45	315

Jackknifed correct attribution = 59.68%

C) Discriminant Analysis averaged by individual

	U. australis	*U. bremii*	*U. intermedia*	*U. minor*	*U. ochroleuca*	*U. stygia*	*U. vulgaris*	Total
U. australis	5	2 (S)	0 (S)	0 (S)	3 (S)	0 (S)	0 (NS)	10
U. bremii	0 (S)	5	1 (S)	2 (NS)	2 (S)	0 (S)	0 (S)	10
U. intermedia	1 (S)	0 (S)	7	1 (S)	0 (S)	0 (S)	0 (S)	9
U. minor	0 (S)	3 (NS)	1 (S)	4	1 (S)	1 (S)	0 (S)	10
U. ochroleuca	0 (S)	0 (S)	0 (S)	0 (S)	3	1 (NS)	0 (NS)	4
U. stygia	0 (S)	0 (S)	1 (S)	1 (S)	0 (NS)	8	0 (S)	10
U. vulgaris	1 (S)	2 (S)	2 (S)	2 (S)	2 (NS)	0 (S)	1	10
Total	7	12	12	10	11	10	1	63

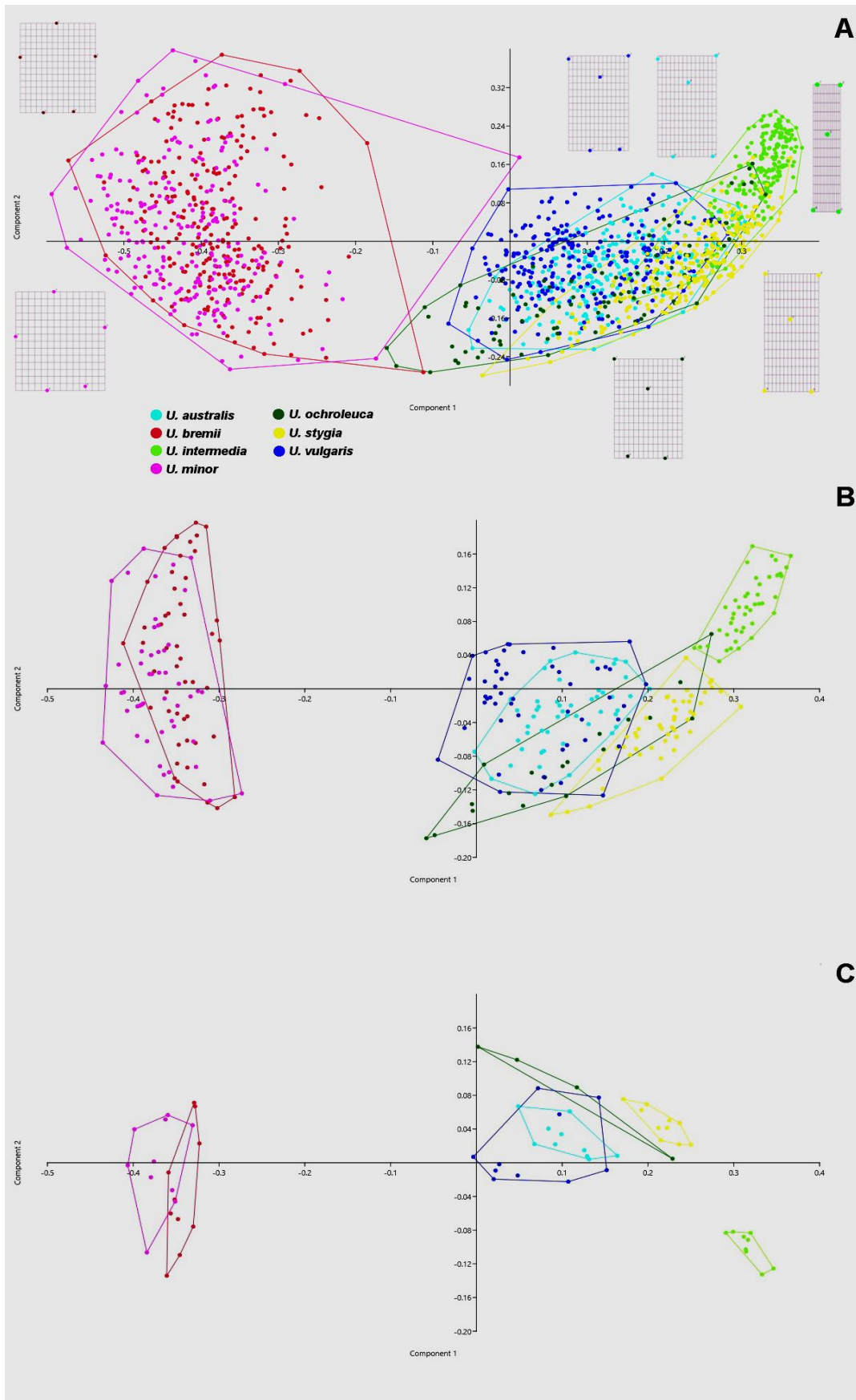Jackknifed correct attribution = 52.38%

Figure **14.** PCA analysis on quadrifid glands of all species. A) All glands analysis with consensus shape of each species, B) analysis averaged by traps and C) analysis averaged by individuals.

Utricularia intermedia aggregate

In the PCA plot (Fig. 15), species within this aggregate show a certain degree of distinction, despite the overlapping of values. The variance explained by the first two axes is respectively 63% ca. and 22% ca. Procrustes ANOVA resulted significant ($P < 0.01$) with both gland and trap as random effect, allowing to average measurements by traps and individuals. Jackknifed confusion matrix calculated with DA gave 70.4% of correct classification. Permutation test with 10000 runs performed pairwise resulted significant for all pairs of species. PCAs of measurements averaged by both traps and individuals show that a separation exists between taxa better than using all glands (Figs. 15B and 15C), especially for *U. intermedia*. This was also confirmed by DA, where jackknifed percentage of correct classification raises to 76.5% in trap averaged analysis and to 78.3% in individual averaged analysis. Permutation test resulted significant for all pairs when measurements were averaged by traps, while, when measurements were averaged by individuals, it resulted not significant for *U. ochroleuca*/*U. stygia* (Table 6).

Utricularia minor aggregate

PCA was not able to separate species as shown by Fig. 16 where values of the two species massively overlap. Variance explained by the first two axes is 40% ca. and ca. 22%. Quite high percentages are found up to the seventh axis. Procrustes ANOVA resulted significant with both gland and trap as random effect. Jackknifed confusion matrix calculated with DA gave 66% ca. of correct classification. Permutation test with 10000 runs performed pairwise resulted significant. PCAs measurements averaged by both traps and individuals do not seem to improve species' separation. Jackknifed percentage of correct classification (DA) respectively increases to 62% when measurements were averaged by traps, to 70% when measurements were averaged by individuals. Permutation test (with 10000 runs) resulted significant when measurements were averaged by traps, while resulted only slightly significant ($T^2 = 0.0185$) when measurements were averaged by individuals (See Table 7).
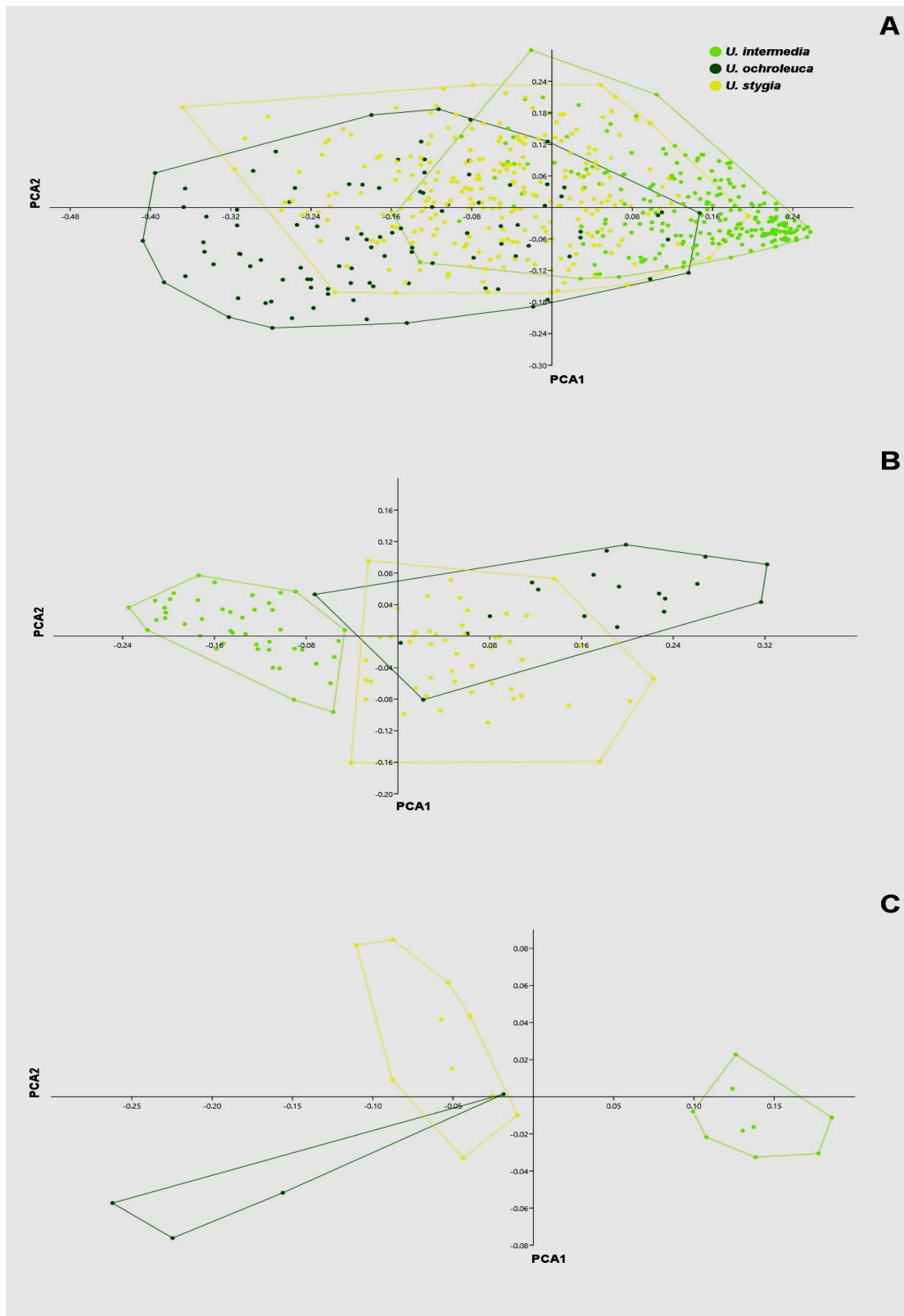
Table 6. Discriminant Analysis (allocation) and permutation test (significance reported in bracket, S = significant at 0.01 level, NS = not significant) on Procrustes coordinates in *U. intermedia* aggr.

A) Discriminant Analysis all glands

	U. intermedia	*U. ochroleuca*	*U. stygia*	*Total*
U. intermedia	173	17 (S)	35 (S)	225
U. ochroleuca	8 (S)	67	25 (S)	100
U. stygia	34 (S)	51 (S)	165	250
Total	215	135	225	575

Jackknifed correct attribution = 70.43%

B) Discriminant Analysis averaged by trap

	U. intermedia	*U. ochroleuca*	*U. stygia*	*Total*
U. intermedia	34	6 (S)	5 (S)	45
U. ochroleuca	3 (S)	15	2 (S)	20
U. stygia	4 (S)	7 (S)	39	50
Total	41	28	46	115

Jackknifed correct attribution = 76.52%

C) Discriminant Analysis averaged by individual

	U. intermedia	*U. ochroleuca*	*U. stygia*	*Total*
U. intermedia	8	1 (S)	0 (S)	9
U. ochroleuca	1 (S)	3	0 (NS)	4
U. stygia	1 (S)	2 (NS)	7	10
Total	10	6	7	23

Jackknifed correct attribution = 78.26%

Utricularia vulgaris aggregate

PCA was not able to separate species, which clearly overlap (Fig. 17). Variance explained by the first two axis is 39% ca. and 27% ca; quite high percentages are found up to the seventh axis. Procrustes ANOVA resulted significant with both gland and trap as random effect. DA confusion matrix gave a jackknifed percentage of 58.2%. Permutation test with 10000 runs performed pairwise resulted significant. A better separation of values is found in averaged analyses, especially when measurements were averaged by individuals. DA confusion matrix calculated for measurements averaged by traps gave a jackknifed percentage of 58%, while for measurements averaged by individuals the percentage increases to 70%. Permutation test (with 10000 runs) resulted significant for analysis with measurements averaged by traps, while not significant ($T^2 = 0.065$) with measurements averaged by individuals (Table 8).
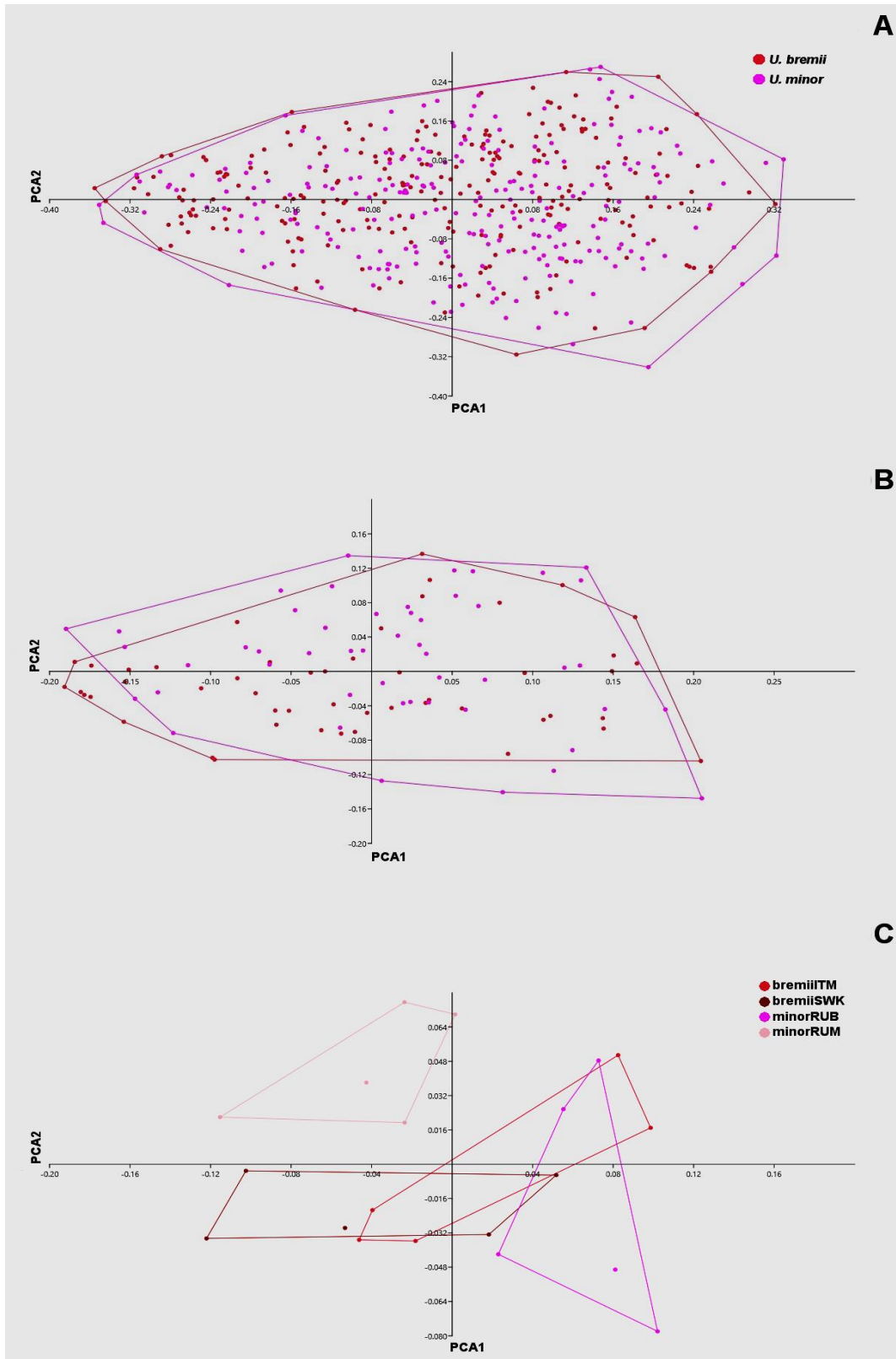
Figure 15. PCA analysis on quadrifid glands of *U. intermedia* aggr. A) All glands analysis, B) analysis averaged by traps and C) analysis averaged by individuals.

Table 7. Discriminant Analysis (allocation) and permutation test (significance reported in bracket, S = significant at 0.01 level, NS = not significant) on Procrustes coordinates in *U. minor* aggr.
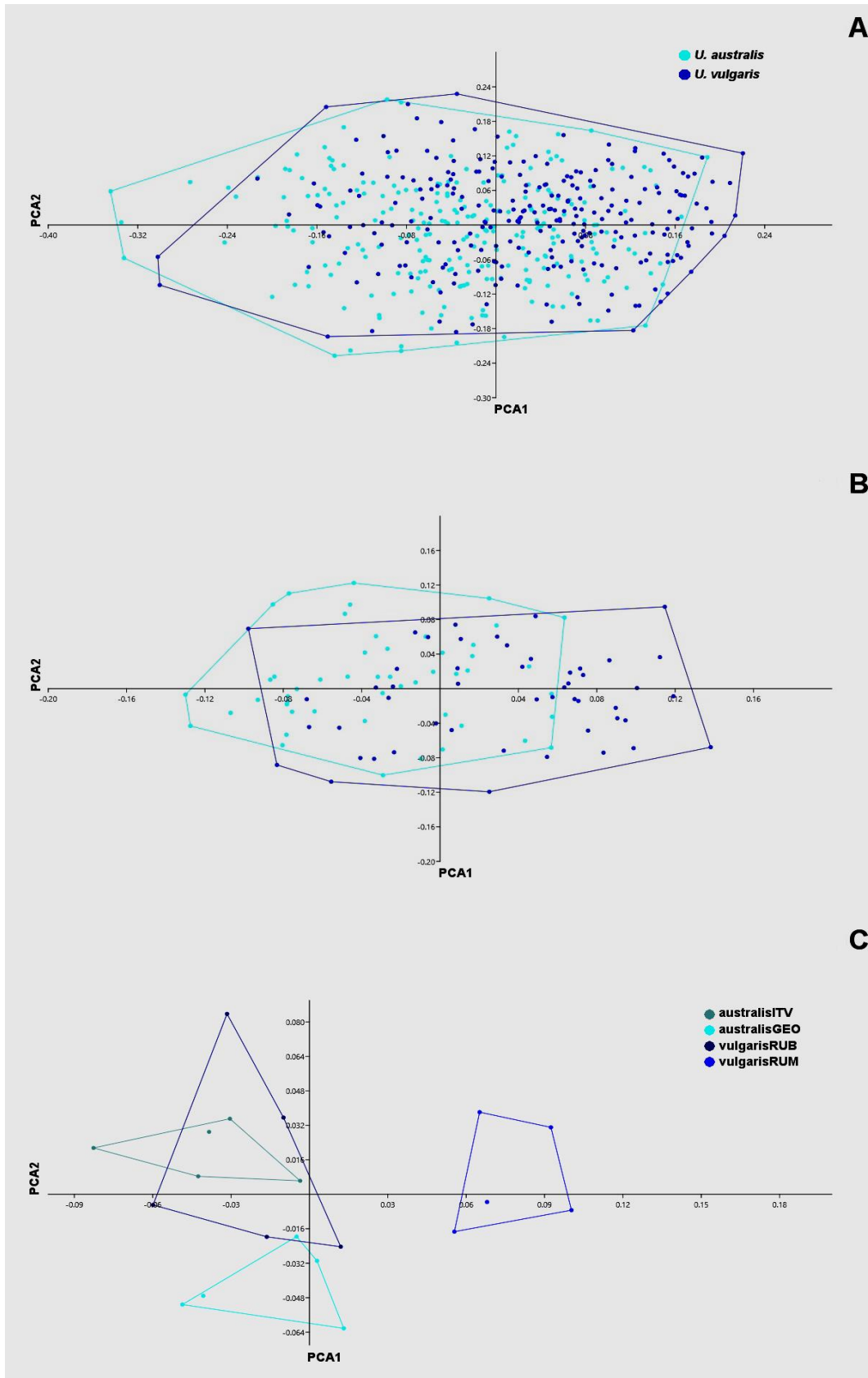
A) Discriminant Analysis all glands

	U. bremii	*U. minor*	Total
U. bremii	144	106 (S)	250
U. minor	110 (S)	140	250
Total	254	246	500
Jackknifed correct attribution = 56.80%			

B) Discriminant Analysis averaged by trap

	U. bremii	*U. minor*	Total
U. bremii	32	18 (S)	50
U. minor	20 (S)	30	50
Total	52	48	100
Jackknifed correct attribution = 62.00%			

C) Discriminant Analysis averaged by individual

	U. bremii	*U. minor*	Total
U. bremii	8	2 (NS)	10
U. minor	4 (NS)	6	10
Total	12	8	20
Jackknifed correct attribution = 70.00%			

Table 8. Discriminant Analysis (allocation) and permutation test (significance reported in bracket, S = significant at 0.01 level, NS = not significant) on Procrustes coordinates in *U. vulgaris* aggr.

A) Discriminant analysis all glands

	U. australis	*U. vulgaris*	**Total**
U. australis	145	105 (S)	250
U. vulgaris	104 (S)	146	250
Total	249	251	500
Jackknifed correct attribution = 58.20%			

B) Discriminant analysis averaged by trap

	U. australis	*U. vulgaris*	**Total**
U. australis	31	19 (S)	50
U. vulgaris	23 (S)	27	50
Total	54	46	100
Jackknifed correct attribution = 58.00%			

C) Discriminant analysis averaged by individual

	U. australis	*U. vulgaris*	**Total**
U. australis	9	1 (NS)	10
U. vulgaris	5 (NS)	5	10
Total	14	6	20
Jackknifed correct attribution = 70.00%			

Figure 16. PCA analysis on quadrifid glands of *U. minor* aggr. A) All glands analysis, B) analysis averaged by traps and C) analysis averaged by individuals.

Figure 17. PCA analysis on quadrifid glands of *U. vulgaris* aggr. A) All glands analysis, B) analysis averaged by traps and C) analysis averaged by individuals.

Size

Centroid size does not clearly discriminate species, even if significant differences were found among species medians (p < 0.01). As shown in Fig. 20, boxplots of the species clearly overlap. Generally, *U. australis* has the smallest glands, while *U. stygia* the largest ones. However, significant differences were found also within species, i.e. within populations of *U. australis*, *U. stygia* and *U. vulgaris* (not shown).
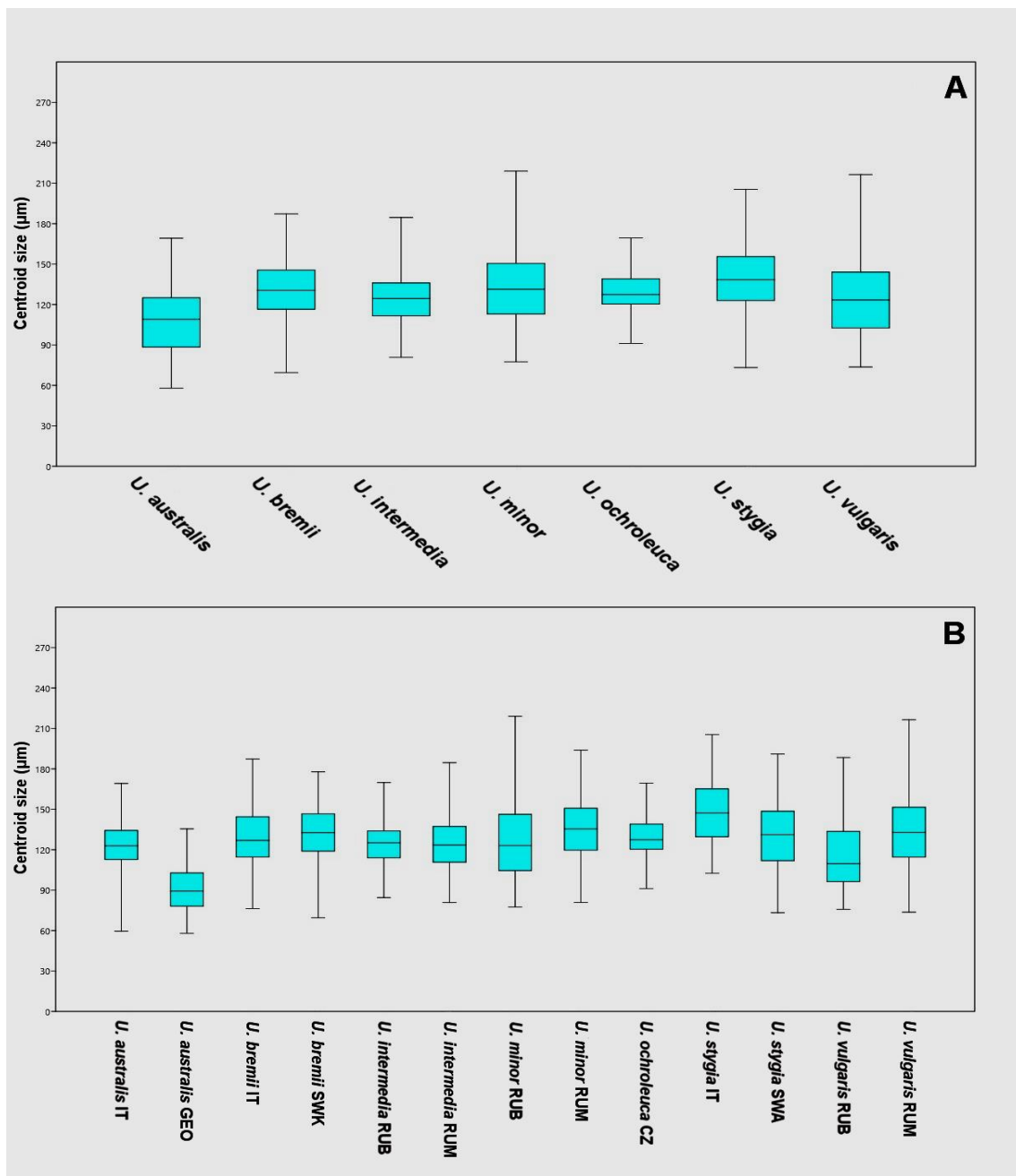
Figure 18. Centroid size (μm) of quadrifid glands in all species. A) Species and B) single populations.

Allometry

In most of the populations allometry had no effect on shape variation, except for *U. vulgaris* RUB (p = 0.00294), marginally for *U. vulgaris* RUM (p = 0.0136) and *U. australis* GEO (p = 0.0294). I performed a PCA using residuals of regression of shape onto size, but the general figure does not change respect to PCA with Procrustes coordinates and the plots are very similar between each other (data not shown). Also DA does not change significantly if residuals are used.

Molecular analysis

DNA Barcoding

Sequence length ranges from 817 bp to 825 bp for *rps*16 intron and from 378 bp to 391 bp for *trn*L-F IGS.

Species of *U. intermedia* aggr. generally share the same haplotypes for both plastidial markers investigated. Three accessions belonging to this aggregate differ for only one substitution or indel nucleotide site, i.e. *intermediaRUB4*, *stygiaITM1*, *stygiaITM2* and *stygiaSWA4*.

Most of the accessions of *U. bremii* share the same haplotypes with one exception: *bremiiITM5*, which is close to most of *U. minor* accessions, differing from these only for 2 or 3 nucelotide sites. Just one accession of *U. minor*, *minorITT2*, is distant from all the other ones of the same species and its closest haplotypes are those of *U. bremii*, being different from 2 to 5 sites.

Concerning *U. vulgaris* aggr., all accessions are relatively close to each other, but accessions of *U. australis* from Viareggio differ from all the other accessions by 5 to 6 sites.

With the exception of the two accessions, *bremiiITM5* and *minorITT2*, haplotypes of *U. bremii* and *U. minor* are distinct, differing by more than 20 sites (including indels).

ITS sequences range from 560 bp to 614 bp in length. No polymorphic site was found in the ITS sequence of any sample.

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 to that of *U. intermedia*.

Concerning *U. vulgaris* aggr., no diagnostic differences were found between accessions of the two species.

**Phylogenetic relationships**

In both phylogenetic plastidial networks and trees, European species are divided in 4 well-separated clades (Figs. 19 and 21).

By combining both plastidial markers, an alignment of 1295 sites was produced. Maximum Parsimony produced, either considering gaps as a fifth state or as missing data, 9 equally most parsimonious trees. In the analysis with gaps treated as fifth state, of 1295 characters, 816 are constant, 367 are variable parsimony-uninformative characters and 112 are parsimony-informative.

In clade I, corresponding to sequences of *U. vulgaris* aggr. and a sequence of *U. macrorhiza*, the relationships between the different species and populations are unresolved, except for *U. australis* from Viareggio, resulting separated from the others sequences but with a quite low support. Clades II and IV are mainly constituted by one species, respectively *U. bremii* and *U. minor*, with no differentiation between populations, while clade III includes all species of *U. intermedia* aggr., but their relationships are unresolved (all the populations of all species bearing the same haplotypes).

Concerning ITS phylogenetic network (Fig. 20), three main clades were found among European species. Clade I is constituted by species of *U. vulgaris* aggr., clade II by sequences of *U. intermedia* and one sccession of *U. ochroleuca* and finally, clade III, includes the rest of the accessions. This latter clade is the most diverse, with accessions belonging to *U. intermedia* aggr. and *U. minor* aggr. distributed among more or less different rybotypes. In some case, within the same rybotype, accessions of different species from different aggregates can be found. Moreover, the relationships between these three clades are not well-defined, even if clade II and III appear closer to each other than to clade I.
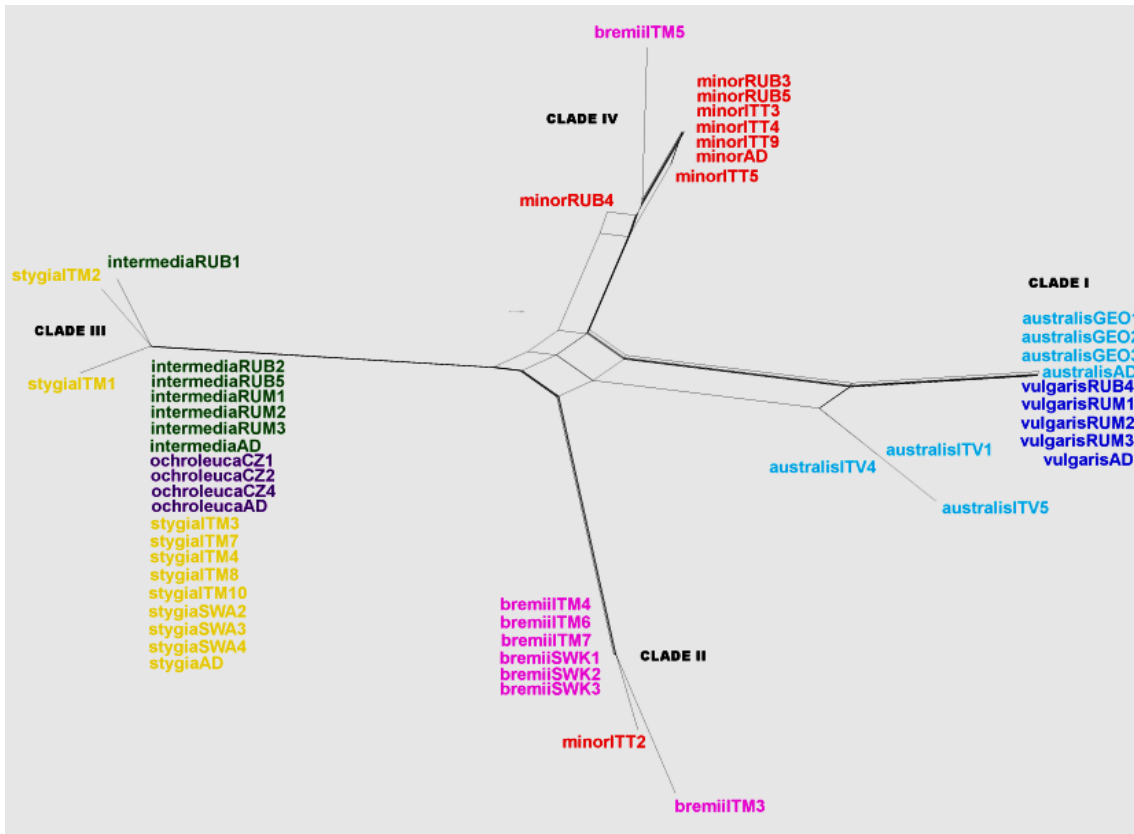
Figure 19. NeighbourNet plastidial *rps*16 intron and *trn*L-*trn*F IGS combined network. Characters transformation based on uncorrected p distances method. AD = Lubomír Adamec personal collection (Třeboň Basin, Czech Republic).
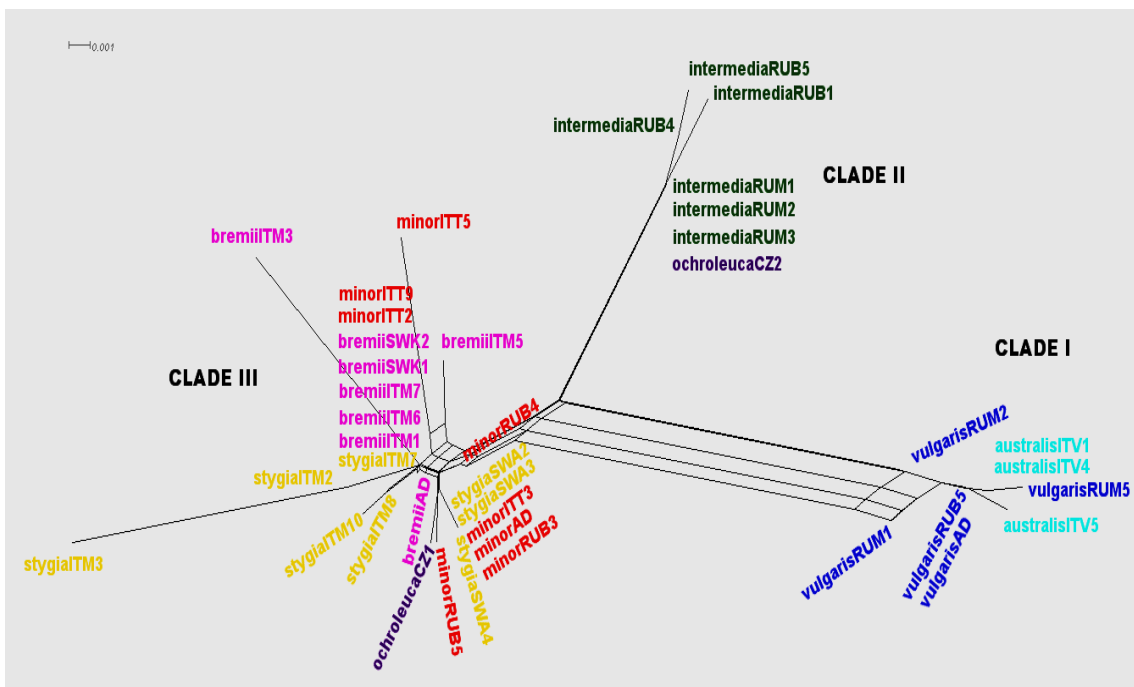
Figure 20. Neighbour-Net ITS. Characters transformation based on uncorrected p distances method. AD = Lubomír Adamec personal collection (Třeboň Basin, Czech Republic).

Figure 21. Plastidial markers *rps*16 intron + *trn*L-F combined alignment. Maximum Parsimony phylogenetic tree. Gaps treated as fifth state. Consensus tree of 10 equally most parsimonious tree. Bootstrap with 10000 pseudoreplicates according to 50% consensus majotiry rule. Bootstrap values lower than 50% not indicated.* = sequences taken from GenBank. Outgroup sequences in black.

DISCUSSION

Morphometric analysis

Generally, this study confirmed the difficulties to find reliable vegetative morphological features able to discriminate European species of *Utricularia*. Thor (1988) proposed an identification key using only features of quadrifid glands for discriminating the six species of *Utricularia* occurring in Scandinavia. Geometric morphometrics should be even more exhaustive than features evaluated by that author (i.e. angle between short arms, angle between long arms, angle between long and short arms and arm length ratio), since it avoids the calculation of many parameters, but considers all of them in just one-step. Despite this, using geometric morphometrics on quadrifid gland shape did not allow any species discrimination. However, it is noteworthy to remember that in Scandinavia, the situation is simpler, since *U. bremii* does not occur, preventing possible confusion with *U. minor*.

Concerning the *U. intermedia* aggr., results showed that teeth on the leaf margin appear to be as a good feature for species discrimination, also considering the possibility to combine this feature with others such as the angle on the tip of the leaf segments. The latter character alone does not seem to be sufficient for species identification, while in the combined analysis, it contributes to recognise species (Fig. 14). In my study, *U. ochroleuca* and *U. stygia* show some intraspecific variability concerning the number of teeth, while *U. intermedia* seems to be less variable, with no statistically significant differences among populations. In addition, also geometric morphometric analysis can be combined with 'traditional' morphometric analysis, in order to distinguish species. Indeed, as shown by PCA in Fig. 17, an overlap between glands' shape exists, but a certain separation of species can be found, especially when considering averaged values (Fig. 17B and 17C). Thus, to identify a non-flowering specimen belonging to one of the species within this aggregate, some valuable tools, eventually to be combined each other, are now available. Few studies were devoted to the quadrifid glands analysis after Thor's (1988) contribution, and they were mainly focused on discussing the validity of the separation between *U. ochroleuca* and *U. stygia* (Schlosser 2003, Płachno & Adamec 2007). In these studies, arm length and angles between the arms were evaluated and the variability existing among populations of both *U. ochroleuca* and *U. stygia* have been highlighted. Concerning angles between shorter arms, both Schlosser (2003) and Płachno & Adamec

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(2007) criticized Thor for providing mean values delimiting species (171° ± 25° for *U. ochroleuca* and 74° ± 22° for *U. stygia*) not corresponding to values found globally, but only attributable to Scandinavian populations. For instance, Schlosser (2003) found a population of *U. ochroleuca* in Czech Republic bearing mean values of 85° ± 26° and a population of *U. stygia* from the USA with mean values of 86° ± 26°. However, Płachno & Adamec (2007) provided an identification key, but only for Czech specimens, while Schlosser (2003) simply highlighted the large intraspecific variability and a much lower difference between the mean angles of the two species than indicated by Thor (1988). My study fully confirms this trend: the three species overlap concerning glands' shape in all glands analysis, but in both averaged analyses (by traps and by individuals) *Utricularia intermedia* is well distinct from the others, which still overlap. The typical quadrifid glands of *U. intermedia* bear four more or less parallel arms (the angle between short arms is close to 0°), but also in this species a large variability even within the same trap was found, as already reported for the other two species belonging to this aggregate (Schlosser 2003, Płachno & Adamec 2007) and largely confirmed here. Another interesting point stressed by Płachno & Adamec (2007), is that different growth conditions may influence size and shape of glands. Indeed, these authors found differences in quadrifid glands between cultivated and natural specimens of the same population. Therefore, also the intraspecific difference may be explained by different ecological conditions in which populations live. For instance, it is commonly known that teeth on leaf margin could vary greatly depending on leaf width, which in turn markedly depends on ecological habit of the plants (submerged/terrestrial and sunny/shady habitats). If so, a special care must be taken to interpret the morphometric data. In this study, I have pooled data for *U. intermedia* and *U. minor* aggregates which include plants of unknown ecological habit. Therefore, my data include a relatively high variability for each species studied which could have been much lower if I had separately used only terrestrial and submerged shoots for analyses. Similarly, as found recently for three European *Utricularia* species, quadrifid gland size depends significantly on trap size (L. Adamec, unpublished data), which could also increase the variability in glands size evidenced by the present study.

Regarding *U. minor* aggr., it was possible to confirm that *U. bremii* generally has quadrifid glands larger than *U. minor*, but the difference is very slight and does not allow a clearcut species identification, not supporting what reported by Tison & de Foucault

(2014). As concerns the shape of the quadrifid glands, the two species resulted very similar, and intraspecific variability was higher than interspecific variability. Indeed, as shown by PCA (Fig. 18C), the population of *U. minor* from Lake Bezymannoye is closer to the two populations of *U. bremii* than to the co-specific population from Lake Michurinskoye.

Regarding *U. vulgaris* aggr., I looked at the ratio between teeth and setula length, as suggested by Thor (1988). If *U. vulgaris* RUM (Lake Michurinskoye) is excluded from the analysis, this character allows a clear discrimination. It is noteworthy to say that I found no flowers at the collection time of plants from Lake Michurinskoye. I attributed these plants to *U. vulgaris* based on flowers found the previous year, all belonging to this species, and on the fact that in the past only *U. vulgaris* had been reported for this site. However, it cannot be excluded that *U. australis* and *U. vulgaris* co-occur there and that eventually I collected both. Except for this population, generally *U. vulgaris* bears a larger ratio than *U. australis*, which appears somehow more toothed than the latter species. We can state that this feature can help to distinguish non-flowering robust individuals within *U. vulgaris* aggr., but it would be even more resolutive if paired with another character, such as the rhizoid shape or turion (winter buds) size and shape, if and when these portions of the plants are available. For instance, it is rare to find herbarium specimens with turions and rhizoids and they are often in bad conditions. More insights deserve the population collected in Lake Michurinskoye, which bears intermediate values between *U. australis* and *U. vulgaris*, possibly representing a hybrid between these two species, both widely occurring in North-western Russia. However, such a hybrid has never been recorded. In past experiments, 74 flowers of *U. australis* were crossed with pollen of *U. vulgaris*, but no seeds were obtained (Beretta et al. 2014). Nevertheless, Beretta et al. (2014) reported for *U. australis* the occurrence of well-developed pollen grains, differently from other sterile species, which bear malformed grains. Thus, the cross between pollen of *U. australis* and flowers of *U. vulgaris* is yet to be tested. Quadrifid gland analysis did not find significant differences between the two species, contrarily to what stated by Thor (1988), who described angles between shorter arms in *U. vulgaris* as lesser than in *U. australis*. Moreover, an intraspecific higher than interspecific variability was found, as shown by PCA. Indeed, *U. vulgaris* RUB is closer to *U. australis* populations than to the co-specific population in Lake Michurinskoye (Russia) (Fig. 19C).

Molecular analysis

Concerning barcoding, different haplotypes/ribotypes were found within the same species or different species shared the same haplotypes/ribotypes. For this reason, it was not possible to use a barcoding approach to discriminate all species. However, by means of ITS marker, discrimination between *U. intermedia* and *U. stygia* is possible.

Within *U. minor* aggr. two different haplotypes were found, almost perfectly corresponding to the two different species. Indeed, just one out of the 9 accession of *U. bremii* clustered with *U. minor*, and one out of the 9 accessions of *U. minor* clustered with *U. bremii*. Thus, a barcoding approach can be applied for distinguishing *U. bremii* and *U. minor*, considering both *rps*16 intron and *trn*L-F IGS markers alignment, but with a little chance of misleading results.

From a phylogenetic point of view, the putatively 'pure' sexual species, i.e. *U. intermedia*, *U. minor* and *U. vulgaris* (Taylor 1989), appear well separated from each other in both plastidial and nuclear networks.

Although most of the accessions referred to *U. minor* cluster together, some genetic variation does exist within this species (accession *minorITT2*, see above). Also *U. bremii* (specimen *ITM5*) displays a trend of genetic variation similar to what found in *U. minor*. As shown in Figs. 19 and 20, incongruences between plastidial and nuclear networks were found, mostly for the sterile species *U. ochroleuca* and *U. stygia*, supporting the hypothesis of their hybrid origin (Neuman 1900, Thor 1988, Schlosser 2003, Płachno & Adamec 2007).

Most of the sequences of *U. bremii* in the combined plastidial network cluster separately from all the other sequences, while in the ITS network they are close to both *U. minor* and *U. stygia*. Thus, only the ITS marker is consistent with the morphological similarity between *U. bremii* and *U. minor*. Probably, if a hybridization event was involved in the origin of *U. bremii*, with *U. minor* as one of the parents, the latter species may have been the male parental species, if plastid in Lentibulariaceae is maternally inherited as in the majority of angiosperms. Nevertheless, also considering the results found in the present study, *U. bremii* does not show morphological characters intermediate between different species, but rather it shows morphological features matching with those of *U. minor*. In addition, with this latter species *U. bremii* also share the same ribotypes and, sometimes, the same haplotypes. For these reasons, the dysploid apomict hypothesis raised by Taylor

(1989) has its validity and the delimitation of *U. bremii* as a different species from *U. minor* remains arguable.

As concerns *U. stygia*, its sequences cluster with the other species of *U. intermedia* aggr. considering the plastidial network, while in the ITS network these sequences cluster close to *U. minor* and *U. bremii* sequences, supporting a putative hybridization *U. intermedia* × *U. minor*, even if fruits of *U. intermedia* have been rarely found (see above).

More puzzling is the situation of *U. ochroleuca*, which is close to the other species of the *U. intermedia* aggr. in plastidial network, while in ITS network the only two sequences available cluster distant from each other, with one accession (*ochroleucaCZ1* from Nadejski fishpond, Třeboň Basin, Czech Republic) resulting close to *U. minor*, and the other accession (*ochroleucaCZ2* from Ptaci Blato, Třeboň Basin, Czech Republic) clustering with *U. intermedia*. Then, if there is evidence of possible hybridization origin for *U. ochroleuca* from Nadejski fishpond, there is not for *U. ochroleuca* from Ptaci Blato. The population from Nadejski fishpond could be derived from a hybridization event *U. intermedia* × *U. minor*. Alternatively, both accessions of *U. ochroleuca* may be of hybrid origin *U. intermedia* × *U. minor*, but the two sequences resulted distant in the network because one of the two individuals retain a copy of the ITS inherited by the male parental species (presumably *U. minor*), while the other retained the copy inherited by the female parental species (presumably *U. intermedia*).

Without claiming hybridization, all these incongruencies between the two different networks may be also explained by incomplete lineage sorting (Doyle 1992, Maddison 1997, Posada & Crandall 2001, Naciri & Linder 2015). Indeed, ITS may be present in different alleles in *U. ochroleuca* populations and different isolated populations may have different copies, with some of these copies being ancestral. For instance, the individual from Ptaci Blato could have retained a copy of the ITS similar to that of *U. minor* and *U. stygia*, maybe being not much differentiated by the ancestral copy present before the separation of *U. intermedia* aggr. and *U. minor aggr.*, while the individual from Nadejski fishpond could have a derivative copy similar to that of *U. intermedia*, originated after the separation of *U. intermedia* aggr. and *U. minor* aggr. In this case we should admit that also *U. stygia* retained an ancestral copy of the ITS. Similarly, the populations of *U. stygia* investigated and maybe the whole species, may have retained a copy of the ITS not much differentiated by the ancestral copy present before the separation of *U. intermedia* aggr.

and *U. minor aggr.*, then resulting close to the latter species in nuclear network and trees, but actually being related to *U. intermedia*.

Sequences of *U. australis* and *U. vulgaris* cluster together as expected considering their morphological similarity, but in plastidial network *U. australis* from Viareggio, Italy, is separated from all other sequences, including the co-specific ones from Oranienbaum Heide, Germany and from Třeboň, Czech Republic (Fig. 19), which are closer to *U. vulgaris* specimens. Then, also for *U. australis* some intraspecific genetic variation exists and is consistent with the hypothesis that each population may represent an apomict unit, not only with a peculiar chromosome number (Taylor 1989), but also with a peculiar genomic profile.

Plastidial phylogenetic tree is in accordance with the network. Indeed, the four main clusters found in the network are recognised and are all well supported clades, but the relationship between them is unresolved.

Looking at the relationships with other species non-native to Europe, *U. gibba* resulted sister to all European species in the plastidial tree, contrasting what found by Rahman (2006, 2007), who related it to *U. bremii*, and confirming what already found by Jobson & Albert (2002) with the same plastidial markers. However, *U. gibba* sequences resulted highly divergent from the rest of the sequences, showing a high substitution rate as shown by the long branch in NJ phylogenetic tree (not shown). In addition, in the studies of Rahman (2006, 2007), *U. intermedia* and *U. minor* resulted very closely related, while in my study the former species shows no clear relationships with neither *U. minor* nor *U. bremii*, which are closely related each other, at least in the ITS phylogeny.

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CONCLUSIONS

European species of *Utricularia* are difficult to distinguish without flowers, but some features of vegetative parts may help for this task. Geometric morphometrics on quadrifid glands did not allow a clear-cut distinction when all species are treated together, but may be of help in the case of species within the *U. intermedia* aggregate, particularly if combined with other 'traditional' morphometric analyses. However, all species in *U. intermedia* aggr. show intraspecfic variability for all characters investigated, including quadrifid glands shape, which display a wide range of variation (Fig. 22). Barcoding with *trn*L-*trn*F IGS and *rps*16 intron revealed unapplicable for most of the critical species, but may be useful for the distinction of *U. bremii* and *U. minor*, even if a little proportion of the halotypes (barcodes) found in *U. bremii* can match the haplotypes (barcodes) found in *U. minor* and viceversa. Another molecular marker, i.e. plastidial *rpl*20-*rps*12 IGS, has been successfully used by other authors (Vitor Fernandes Oliveira de Miranda & Saura Rodrigues da Silva, personal communication) in barcoding approach applied to large datasets of *Utricularia* species. This marker could be eventually tested on European species for diagnostic purposes.

The large intraspecific variability found for all analyses and for almost all species here studied may be due to the possible hybrid origin of some of them (Neuman 1900, Thor 1988, Schlosser 2003, Płachno & Adamec 2007). Taylor (1989) provided an alternative explanation for this variability: he doubted about the role of hybridization in the speciation and evolution of the mostly sterile European species, whereas he considered each of these species constituted by several morphologically different vegetative apomicts. The hybrid hypothesis was partially confirmed by molecular analyses in this study, at least for *U. stygia*, and also the occurrence of extant hybrids seems to be supported in *U. ochroleuca*. However, some caution is needed when handling these molecular results, since the possible influence of incomplete lineage sorting and other biases affecting the computation of trees and networks. An enlargement of the dataset both in terms of populations and markers could help to shed light on the hybrid hypothesis.

Concerning *U. bremii*, it would be interesting to extend morphometric analyses to the flower morphology in order to test with a quantitative approach the putative diagnostic features and to validate the status of separate species from *U. minor*.

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 & Albert 2002, Müller et al. 2004), this kind of data must be taken cautiously. The vegetative apomict hypothesis is supported by the different chromosome numbers found within the same species (dysploidy). However, some authors highlighted the difficulties in obtaining reliable chromosomes counts, possibly affecting the chromosome counting. Thus, an improvement of the karyological knowledge would be helpful for shedding light on the dysploid hypothesis and on general relationships between closely related species.

Conclusive remarks derived from the present study are summarized in Fig. 23 and an identification key using only vegetative parts of European species of *Utricularia* is presented below.
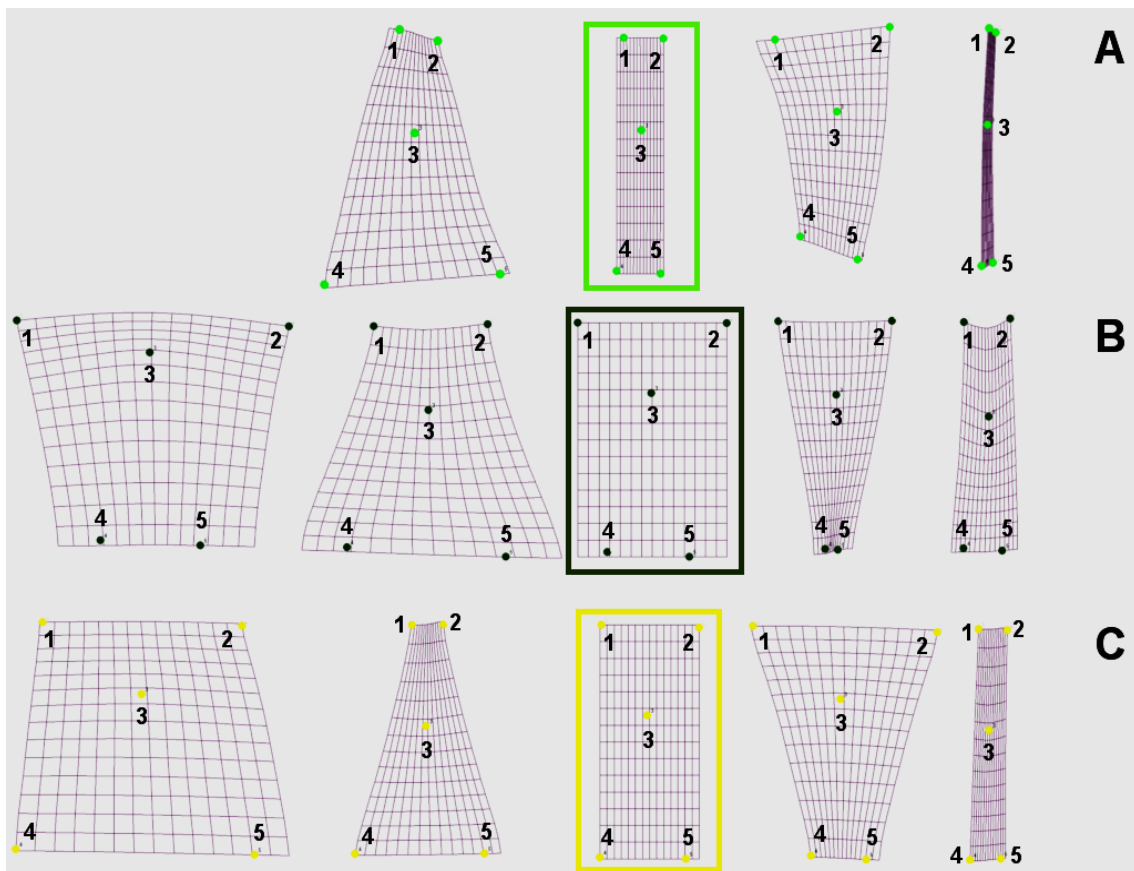
Figure 22. Summary of quadrifid glands of *U. intermedia* aggr.: variability examples of deformation grids among and within the three species. A) *U. intermedia*, B) *U. ochroleuca* and C) *U. stygia*. Consensus configuration of each species in frame.
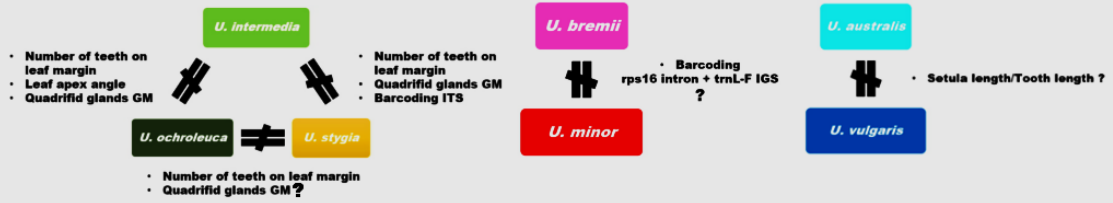
Figure 23. Summary of the results found in this study. H = putative hybridogenous taxa according to the present study.

IDENTIFICATION KEY OF EUROPEAN SPECIES OF *UTRICULARIA* L. USING MORPHOLOGICAL CHARACTERS OF VEGETATIVE PARTS

(For the use of quadrifid glands in *U. intermedia* aggr. it is recommended to investigate at least 5 glands per trap and 5 traps per individual)

1. Leaf segments with margins without teeth bearing setulae . . . .*U. minor* **aggr.**

1. Leaf segments with margins with teeth bearing setulae 2

   2. Dimorphic stolons: green ones above the substrate, pale white ones ± buried in the substrate; leaves on green stolons palmato-dichotomously divided 3

      3. Leaf segments with margins with (8)9 - 16(22) teeth bearing setuale; quadrifid glands shape as in Fig. 22A . . . . *U. intermedia*

      3. Leaf segments with margins with (1)2 - 8(10) teeth bearing setulae; quadrifid glands shape as in Figs. 22B and 22C 4

         4. Leaf segments with margins with (1)2 - 5(6) teeth bearing setuale; quadrifid glands shape variation as in Fig. 22B . . . .*U. ochroleuca*

         4. Leaf segments with margins with 5 - 9(10) teeth bearing setuale; quadrifid glands shape variation as in Fig. 22C . . . . *U. stygia*

   2. Stolons all green growing above the substrate; leaves on green stolons pinnately divided. 5

      5. Setula on the lateral margin of leaf segments (1.70)2.14 - 4.48(6.50) times longer than the tooth from which arises . . . . *U. australis*

      5. Setula on the lateral margin of leaf segments (2.44)3.50 - 17.42(40.20) times longer than the tooth from which arises . . . . *U. vulgaris*

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APPENDIX I

Synonims of European species of *Utricularia*. In this list, names misapplied with other species are not included.

Utricularia intermedia aggr.

U. intermedia Hayne
U. media K.Schum. (1801) in Enum. Pl. Saell. 1: 9.
U. millefolium Nutt. ex Tuckerman (1843) in Amer. J. Sci. & Arts I 14: 28.
U. grafiana Koch (1847) in Flora 30: 265.
Lentibularia intermedia (Hayne) Nieuwl. & Lunell (1917) in Amer. Midl. Nat. 5: 9.

U. ochroleuca R.Hartm.
U. occidentalis A.Gray (1883) in Proc. Amer. Acad. 19: 95.
U. brevicornis Čelak. (1887) in Oesterr. Bot. Zeitschr. 36: 253.
U. × litoralis (*U. intermedia × U. ochroleuca*) Melander (1887) in Bot. Notiser 40: 175
U. intermedia Hayne × *U. minor* L. – Neuman in Bot. Notiser 1900: 65–66.
U. dubia ochroleuca E.H.L.Krause (1903) in Sturm, Fl. Deutschland, ed. 2, 10: 223. nom. illeg.
U. intermedia Hayne f. *ochroleuca* (R.Hartm.) Komiya (1972) in Syst. Stud. Lentib.: 76.

U. minor aggr.

U. bremii Heer
U. pulchella C.B.Lehmann (1843) in Flora 26: 785.
U. minor var. *bremii* (Heer) Franchet (1885) in Fl. Loir-et-Cher: 459.
U. minor subsp. *bremii* (Heer) Bertsch & F.Bertsch (1948) in Fl. Württemburg & Hohenzollern: 386.

U. minor L.
Lentibularia minor (L.) Raf. (1838) in Fl. Tellur. 4: 108.
Xananthes minor (L.) Raf. (1838) in Fl. Tellur. 4: 108.
Utricularia rogersiana Lace (1915) in Bull. Misc. Inf. Kew 1915: 404.
U. minor var. *multispinosa* Miki (1934) in Bot. Mag. Tokyo 48: 337
U. multispinosa (Miki) Miki (1937) in Water Phan. Japan: 109.
U. nepalensis Kitamura (1954) in Acta Phytotax. Geobot. 15: 133.

U. vulgaris aggr.

U. australis R.Br.
U. major Schmidel (1771) in Icones Plantarum ed. Bischof: 80
U. neglecta Lehm. (1828) in Nov. Stirp. Pug. 1: 38.
U. sacciformis Benj. (1847) in Linnaea 20: 302.
U. spectabilis Madauss ex Schreiber (1853) in Arch. Ver. Freunde Naturg. Mecklenb. 7: 233–234.
U. protrusa Hook.f. (1854) in Fl. Nov. Zel. 1: 206.
U. vulgaris L. var. *mutata* Döll (1859) in Fl. Baden 2: 654.

U. vulgaris L. var. *neglecta* (Lehm.) Cosson & Germain (1861) in Fl. Env. Paris, ed.2: 375.

U. pollichii F.Schulz (1871) in Flora 54: 390.

U. mutata (Döll) Leiner (1873) in Arch. Pharm. 2: 46.

U. dubia Rosellini ex Cesati, Passerini & Gibelli (1881) in Comp. Fl. Ital.: 416. nom. illeg.

U. vulgaris f. *tenuis* Saelan (1883) in Medd. Soc. F. & Fl. Fenn. 9: 152.

U. jankae Velen. (1886) in Abh. Konigl. Bohm. Ges. Wiss. VII 1: 37.

U. incerta Kamiénski (1902) in Engler, Bot. Jahrb. 33: 111.

U. mairii Cheeseman (1906) in Man. New Zeal. Fl.: 560.

U. japonica Makino (1914) in Bot. Mag. Tokyo 28: 28.

U. tenuicaulis Miki (1935) in Bot. Mag. Tokyo 49: 847.

U. siakujiiensis Nakajima (1937) in Tokyo Ryocuchi-keikaku Chosa.ihŏ 9: 90.

U. siakujiiensis Nakajima ex Hara (1948) in Enum. Sperm. Jap. 1: 293.

U. vulgaris L. var. *japonica* (Makino) Yamanaka (1953) in Acta Phytotax. Geobot. 15: 32.

U. vulgaris L. var. *formosana* Kuo (1968) in Biol. Bull. Nat. Taiwan Normal Univ. 3: 24.

U. vulgaris L. var. *tenuicaulis* Kuo (1968) in Biol. Bull. Nat. Taiwan Normal Univ. 3: 24.

U. vulgaris L. f. tenuicaulis (Miki) Komiya (1972) in Syst. Stud. Lentib.: 89.

U. australis R.Br. f. *tenuicaulis* (MikiI Koniya & Shibata (1980) in Bull. Nippon Dental Univ., Gen. Educ. No. 9: 48.

U. vulgaris L.

Lentibularia major Gilib. (1781) in Fl. Lituan. 1: 139.

L. vulgaris (L.) Moench (1794) in Meth.: 520.

Utricularia officinalis Thornton (1812) in Brit. Fl. 1: 25.

U. major Cariot & St.Lager (1897) in Bot. Element., ed. 8, 2 Fl. Descr.: 646.

U. vulgaris L. var. *typica* Meister (1900) in Mém. Herb. Boiss. 12: 31.

U. × biseriata H.Lindb. (1921) in Medd. Soc. F. & Fl. Fenn. 13: 1936–1937: 28.

U. intermedia Hayne × *U. vulgaris* L. – Hjelt (1923) in Consp. Fl. Fenn.: 126.

APPENDIX II

Herbarium specimens studied of *U. australis*, *U. ochroleuca* and *U. vulgaris*.

Utricularia australis:—**FINLAND**: Regio Aboënsis, Uusikaupuki, Lepäinen village. Abundantly in pools in abandoned granite quarries: eastern part of Lepäinen island, September 6, 1971, *Unto Laine s.n.* (LE! sub *Utricularia neglecta* Lehm.); **FRANCE**: Eaux stagnantes sur le diluvium de la plaine près de Weissenburg (Alsace), 29 août 1871, *F. Schultz s.n.* (LE! sub *Utricularia neglecta* Lehm.); **GERMANY**: Fl. Hamburg (LE!); **UNITED KINGDOM**: Surrey, England, Wire Mill Pond, 7.1889, *W.F. Miller & Arthur Bennett s.n.* (LE! sub *Utricularia neglecta* Lehm.).

Utricularia ochroleuca:—**FRANCE**: Vosges**,** Dans les mares qui avoisinent le Lac de Longemer près Gérardmer, Août 1868, *S. Perrin s.n.* (FI! sub *Utricularia intermedia*); **POLAND**: Pomerania, Swinemünde, Sumpfwiesen bei Westwine, 3.8.94, *R. Ruthe s.n.* (FI! sub *Utricularia intermedia*).

Utricularia vulgaris:—**DENMARK**: Sattrup Bog. In eutrophic bog with *Nymphaea alba*, *Hydrocharis morsus-ranae*, *Sparganium minimum*, and *Phragmites communis*, 25.7.1967, *L. Holm-Nielsen & S. Jeppesen s.n.* (LE!); **POLAND**: Śląsk Dolny. Rotkitki koło Chojnowa, woj. Legnica. W stawie. *Silesia Inferior.* Roktiki *prope* Chojnów, *palat.* Legnica. *In stagno,* 20 VII 1981, *Edward Kozioł s.n.* (LE!); **AUSTRIA**: Austria inferior, in fodinis prope Seitenstetten, *Strasser s.n.* (LE!); **HUNGARY**: In paludosis ad lacum Fertö tó, prope p. Nersider, 19.VI.1903, *Dr. Filarszky s.n.* (LE!); **CANADA**: Prov. Saskatchewan, Umbgebung von Lloydminster, flacher See bei Maidstone, 3.8.1965, *H. und H. Doppelbaur s.n.* (LE!); **SWEDEN**: Prov. Södermanland, Paroecia Tveta, in sinu lacus Måsnaren prope Varnbäcken, in aqua non profunda, 27 Jul. 1926, *Erik Asplund s.n.* (LE!); **POLAND**: Swinemünde, s.d., *unreadable collector s.n.* (LE!); **BELGIUM**: Prov. D'Anvers, env. Des Malines, fossés, mares, Juillet 1867, *Cap^e Defacqz. s.n.* (LE!); unreadable label *s.n.* (LE!).

PAPERS AND ABSTRACTS (last three years)

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Peruzzi, L., **Astuti, G.**, Carta, A., Roma-Marzio, F., Dolci, D., Caldararo, F., Bartolucci, F. & Bernardo, L. (2015) Nomenclature, morphology, karyology and SEM cypselae analysis of *Carduus brutius* (Asteraceae) and its relatives. Phytotaxa 202: 237-249.

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OTHER PAPERS AND SHORT NOTES:

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Astuti, G., Brullo, S., Domina, G. El Mokni, R. Giordani, T. & Peruzzi, L. (2015) Relazioni filogenetiche tra le specie poliploidi di *Bellevalia* (Asparagaceae) endemiche del Mediterraneo centro-meridionale: risultati preliminari. Scientific meeting of Gruppo per la Floristica, Sistematica ed Evoluzione, Società Botanica Italiana, 20-21/11/2015, Rome.

da Silva, S.R., Varani, A.M., **Astuti, G.**, Peruzzi L., de Moraes, A., Penha, H.A., Aranguren, Y., Menezes, C.G., Michael, T. & de Miranda, F. O. V. (2015) Tamanho de genoma e cariótipo de *Utricularia reniformis* A.St.Hil. (Lentibulariaceae). 66th Brazialian National Congress of Botany, 25-30/10/2015, Santos (SP).

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Astuti, G., Cristofolini, G., Peruzzi, L. & Pupillo, P. (2013) Indagini biosistematiche su alcuni popolamenti di *Pulmonaria officinalis* (Boraginaceae) del Trentino Alto-Adige. Books of abstracts, Scientific meeting of Gruppo per la Floristica, Sistematica ed Evoluzione, Società Botanica Italiana, 18-19/10/2015, Rome.

Astuti, G., Lysak, M., Mandakova, T. & Peruzzi, L. (2013) The mysterious case of *Bellevalia webbiana*: which and how many parents for this rare angiosperm species? Book of abstracts, XIV Optima Meeting, 9-15/09/2013, Palermo.

ACTIVITIES DONE ABROAD

During my PhD program, for 6 months I was visiting student at Saint Petersburg State Univeristy, Russia, where I carried out both field activities for collection of study material and laboratory analysis, under the coordination of Dr. Elena Sabaneyeva at Department of Citology and Histology, within the CINAR Pathobacter Project. Furthermore, for 3 months I was guest at the Laboratory of Systematic Botany, UNESP Jaboticabal, State of São Paulo, Brazil, where I carried out molecular analyses under the supervision of Prof. Vitor Fernandes Oliveira de Miranda.