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ORIGINAL ARTICLE

Functional anatomy of the ovule in *Genlisea* with remarks on ovule evolution in Lentibulariaceae

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Abstract The Lentibulariaceae are highly evolved and specialized carnivorous angiosperms displaying not only unusual morphology and embryology but also specific changes in the genome and chromosomes as large as bacterial chromosomes. Comparative study of the morphology and detailed anatomy of the ovule in the genera *Genlisea*, *Utricularia*, and *Pinguicula* should shed new light on the phylogeny of this family. The clade *Genlisea* + *Utricularia* is sister to the genus *Pinguicula*, which is considered the most primitive taxon within Lentibulariaceae. Thus we should expect the ovules of *Genlisea* to be more similar to those of the more closely related genus *Utricularia* than to *Pinguicula*. Surprisingly, the ovules of *Genlisea* retain characters (free funiculus, ES remaining in the ovule) in common with *Pinguicula*, presumably inherited from a common ancestor. *Genlisea* ovules have only one main character in common with subgenus *Polypompholyx* (*Utricularia*): a well-developed funiculus. There are differences between the ovules of the subgenera *Genlisea* and *Tayloria*. In subgenus *Genlisea* the micropyle tends to be closer to the funiculus and the ovule forms an unusual jacket-like nutritive tissue of integumental origin. The most specialized ovules in Lentibulariaceae evolved in the genus *Utricularia*. The special chalazal nutritive tissue in *Genlisea* and *Utricularia* is simply a hypostase.

Keywords Ovule · Micropyle · Hypostase · Embryo sac · Ovule evolution · Carnivorous plants · Lentibulariaceae

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Introduction

Embryological characters (gynoecium, ovule shape and structure, mega-, and microgametophyte development) are useful and important in the study of plant phylogeny. Together with molecular and paleontological data, embryological characters can be used to reconstruct the evolutionary history of angiosperms. For this we need broad embryological studies, which are very time-consuming (e.g., see Igersheim and Endress 1998; Endress and Igersheim 2000; Igersheim et al. 2001; Endress 2005). For instance, Hydatellaceae were found to be sister to Nymphaeales by a combined molecular and structural phylogenetic analysis, including also embryological features, and to be relic angiosperms (Saarela et al. 2007; Friedman 2008). According to Endress (2005, p. 750), "Ovules are especially interesting in angiosperm systematics and evolution because their classical features are macrosystematically even more constant than previously assumed." The structure and development of the ovules of basal angiosperms like Amborellaceae, Chloranthaceae, Magnoliales, and Nymphaeales are especially relevant to an understanding of angiosperm phylogeny and have been well studied recently (e.g., Igersheim and Endress 1997; Yamada et al. 2001a, b, 2003). Our knowledge of angiosperm ovule anatomy is still insufficient. The Lentibulariaceae represent highly evolved and specialized carnivorous angiosperms displaying not only unusual morphology ("relaxed morphology", Rutishauser

and Isler 2001; Ellison and Gotelli 2009) but also specific changes in the genome and chromosomes as large as bacterial chromosomes (Müller et al. 2004, 2006; Jobson et al. 2004; Laakkonen et al. 2006; Greilhuber et al. 2006). Here we examine ovules of Lentibulariaceae in detail.

The small genus *Genlisea* comprises perhaps of two dozen species classified in two subgenera: *Tayloria* (three species: *Genlisea lobata* Fromm-Trinta, *Genlisea uncinata* P. Taylor & Fromm-Trinta, *Genlisea violacea* St.-Hil. and some yet-unnamed species such as *Genlisea* sp. 'Itacambira Beauty', all restricted to Brazil) and *Genlisea* (18 species, Afro-American distribution; Taylor and Fromm-Trinta 1983; Fisher et al 2000; Fig. 1). The present range of this genus is from South and Central America to Africa including Madagascar (Fromm-Trinta 1977, 1979; Fisher et al. 2000). In contrast to *Genlisea*, the related genus *Utricularia* (Jobson and Albert 2002; Jobson et al. 2003; Müller et al. 2002, 2004) not only has more complicated vegetative morphology (e.g., Brugger and Rutishauser 1989) but is also much richer in number of species (ca. 223; e.g., see Lowrie et al. 2008) and has a worldwide distribution (Taylor 1989). Unlike in *Utricularia* (e.g., Kamieński 1877, 1878; Merz 1897; Merl 1915; Khan 1954, 1992; Płachno and Świątek 2008), the embryology of *Genlisea* is poorly known and was studied only in the late nineteenth (Kamieński 1890) and early twentieth centuries (Merl 1915).

Comparison of the morphology and detailed anatomy of *Genlisea* ovules with the genera *Utricularia* and *Pinguicula* should shed new light on the evolution of Lentibulariaceae. Molecular studies (Jobson and Albert 2002; Jobson et al. 2003; Müller et al. 2000, 2004) have shown the clade *Genlisea* + *Utricularia* to be sister to the genus *Pinguicula* (Fig. 1), which is considered the most primitive taxon among the Lentibulariaceae family (Taylor 1989; Müller et al. 2006). Thus we should expect the ovules of *Genlisea* to be more similar to those of the more related genus *Utricularia* than to *Pinguicula*. According to some authors, subgenus *Polypompholyx* is the most primitive taxon within the genus

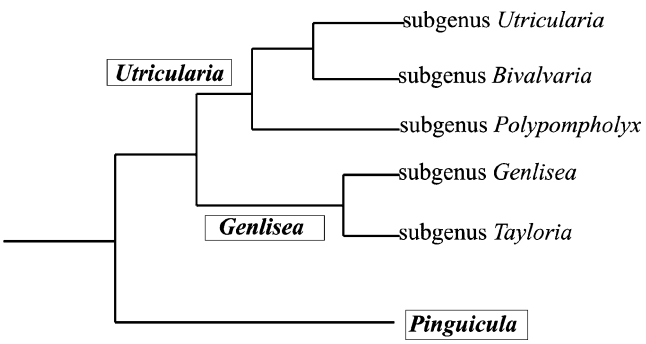

Fig. 1 Simplified cladogram of Lentibulariaceae based on molecular studies by Jobson et al. (2003) and Müller et al. (2004, 2006)

Utricularia (Taylor 1989; Müller and Borsch 2005). Some features of pollen architecture (Taylor 1989; Lobreau-Callen et al. 1999) and trap structure of members of subgenus *Polypompholyx* are similar to *Genlisea* (Reifenrath et al. 2006). Thus the ovules of members of *Polypompholyx* should have characters similar to those in *Genlisea*. In this study we test these two hypotheses.

Materials and methods

Plants of *Genlisea aurea* St.-Hil. (subgen. *Genlisea*, Fisher et al. 2000) were obtained from Chapada dos Guimaraes and Itacambira (Mato Grosso state, Brazil); additional plant material (fixed flowers) was given to us from the Bonn Botanical Garden (accession no. 8546). Flowers of *G. lobata* Fromm-Trinta (subgen. *Tayloria*) were obtained from Espirito Santo (Mato Grosso state, Brazil) and *Genlisea hispidula* Stapf (subgen. *Genlisea*) from Africa. We also examined flowers of the genera *Pinguicula* (subgen. *Isolba*: *Pinguicula agnata* Casper; subgen. *Pinguicula*: *Pinguicula moctezumae* Zamudio & R.Z. Ortega and *Pinguicula moranensis* H.B.K. cultivars) and *Utricularia* (subgen. *Bivalvaria*: *Utricularia sandersonii* Oliv. *Utricularia livida* Mey.; subgen. *Utricularia*: *Utricularia longifolia* Gardner) obtained from the greenhouse collections of Kamil Pásek and from the Botanical Garden of the Jagiellonian University in Cracow, Poland.

Light and electron microscopy

Part of the material was fixed in acetic alcohol, embedded in paraffin, microtome-sectioned 10 μm thick, and stained with Heidenhain's hematoxylin and alcian blue. The PAS reaction was used to detect water-insoluble polysaccharides with 1,2-glycol groups, such as starch (Wędzony 1996). For electron microscopy, placentas with ovules were isolated from ovaries and fixed in 2.5% formaldehyde and 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.0) for 2 days. The material was postfixed in 1% OsO_4 in the cacodylate buffer for 24 h at ~4°C, rinsed in the same buffer, treated with 1% uranyl acetate in distilled water for 1 h, dehydrated with acetone and embedded in Epon 812 (Fullam, Latham, NY, USA). Semithin sections were stained with methylene blue and examined with an Olympus BX60 microscope. Ultrathin sections were cut on a Leica ultracut UCT ultramicrotome. After contrasting with uranyl acetate and lead citrate, the sections were examined in a Hitachi H500 electron microscope. The procedures for preparing samples for SEM were as described earlier (Płachno et al. 2005a, b). Flowers were hand-sectioned with a razor blade and fixed as for TEM.

The dried tissues were sputter-coated with gold and viewed in a HITACHI S-4700 microscope (Scanning Microscopy Laboratory of Biological and Geological Sciences, Jagiellonian University). Additionally, whole ovules were examined with epifluorescence microscope.

Results

G. aurea

G. aurea forms a stalked, approximately spherical basal placenta covered with numerous (several dozen) ovules. The apex of the placenta is sterile and lacks ovules. The placenta consists mainly of highly vacuolated parenchyma cells. Their nuclei do not contain protein inclusions. Placental cells possess small chloroplasts (not shown). The ovule is tenuinucellate and unitegmic, ca. 150 μm long. The integument consists of two or three cell layers. It is partially fused with the funiculus, but the apex of the integument is free (Fig. 2a, b). The free part of the funiculus is prominent. The apex of the integument is curved, so that the micropyle is near the funiculus (Fig. 2b, c). The ovule is anatropous or hemianatropous, however the chalaza, embryo sac, and micropyle lie in a slightly curved line. A few cells of the funicular epidermis may be papillose, probably forming an obturator. A cup-shaped hypostase occurs at the chalazal pole of the ovule, (Figs. 2d and 3a). It directly borders and surrounds the antipodes. The hypostase cell walls are very thick and layered. The nucleus is prominent; the cytoplasm is dense, with small mitochondria. The wall of the antipodals is thin and covered by wall ingrowths (Fig. 3b). Wall ingrowths also occur on the side of the central cell on the border with antipodals. The mitochondria of antipodals are active.

Some cells of the integument have thick, prominent cell walls, and cover the ES near the egg cell at the micropylar pole (Fig. 3c, d), but not at the micropylar apex of the ES (near micropylar part of synergids). These thick-walled cells form a jacket-like structure. The thick cell walls exhibit strong fluorescence under UV light (Fig. 3e, f) and also strong staining with the PAS reaction. The anticlinal walls of these cells are very thick, but they have plasmodesmata (Fig. 4). Plasmodesmata also occur between these cells and the parenchymal cells of the next integumental layer. The thick-walled cells have a prominent nucleus but the cytoplasmic content varies among the cells. Some of them have dense cytoplasm with small lipid bodies or more translucent cytoplasm with various small vesicles or vacuoles (Fig. 4). Between the embryo sac and the integumental cells are collapsed cells with some remains of the protoplast (Fig. 4).

G. hispidula

The ovules are numerous (Fig. 5a). The micropyle is close to the funiculus (Fig. 5b). The ovule is tenuinucellate and unitegmic, ca. 180 μm long. The funiculus is well visible at

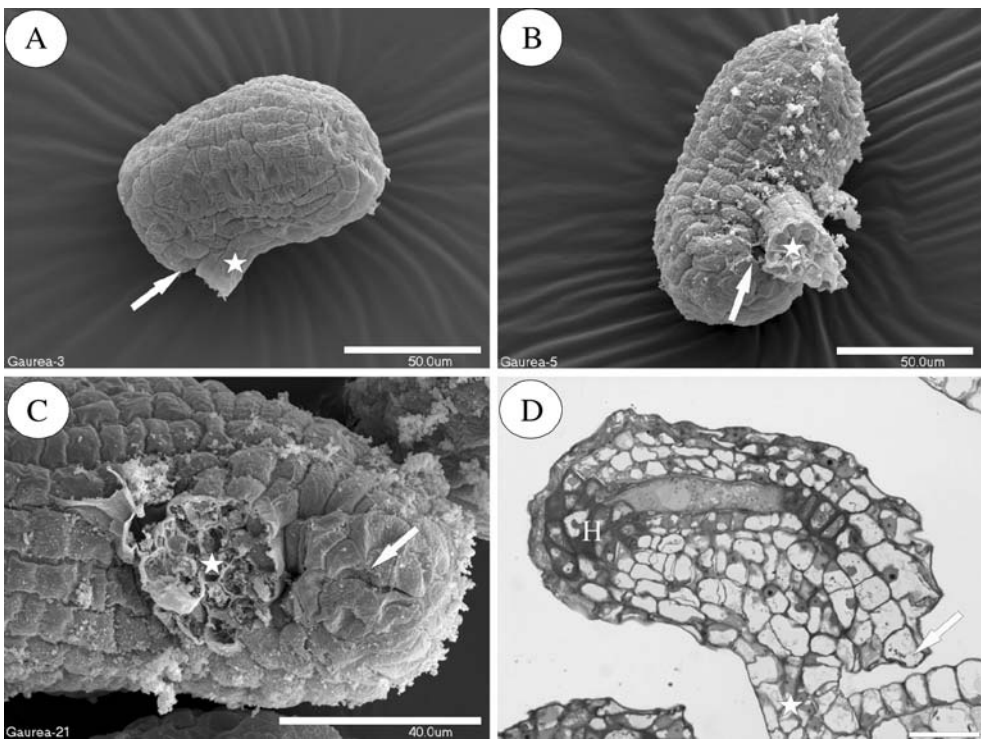

Fig. 2 Morphology and anatomy of ovule of *Genlisea aurea*. **a** and **b** isolated ovule: *arrow* micropyle, *star* funiculus, *bar*=50 μm. **c** Micropyle (*arrow*) adheres to the funiculus (*star*), *bar*=40 μm. **d** Sections through ovule *H* hypostase, *arrow* micropyle, *star* funiculus, *bar*=17 μm

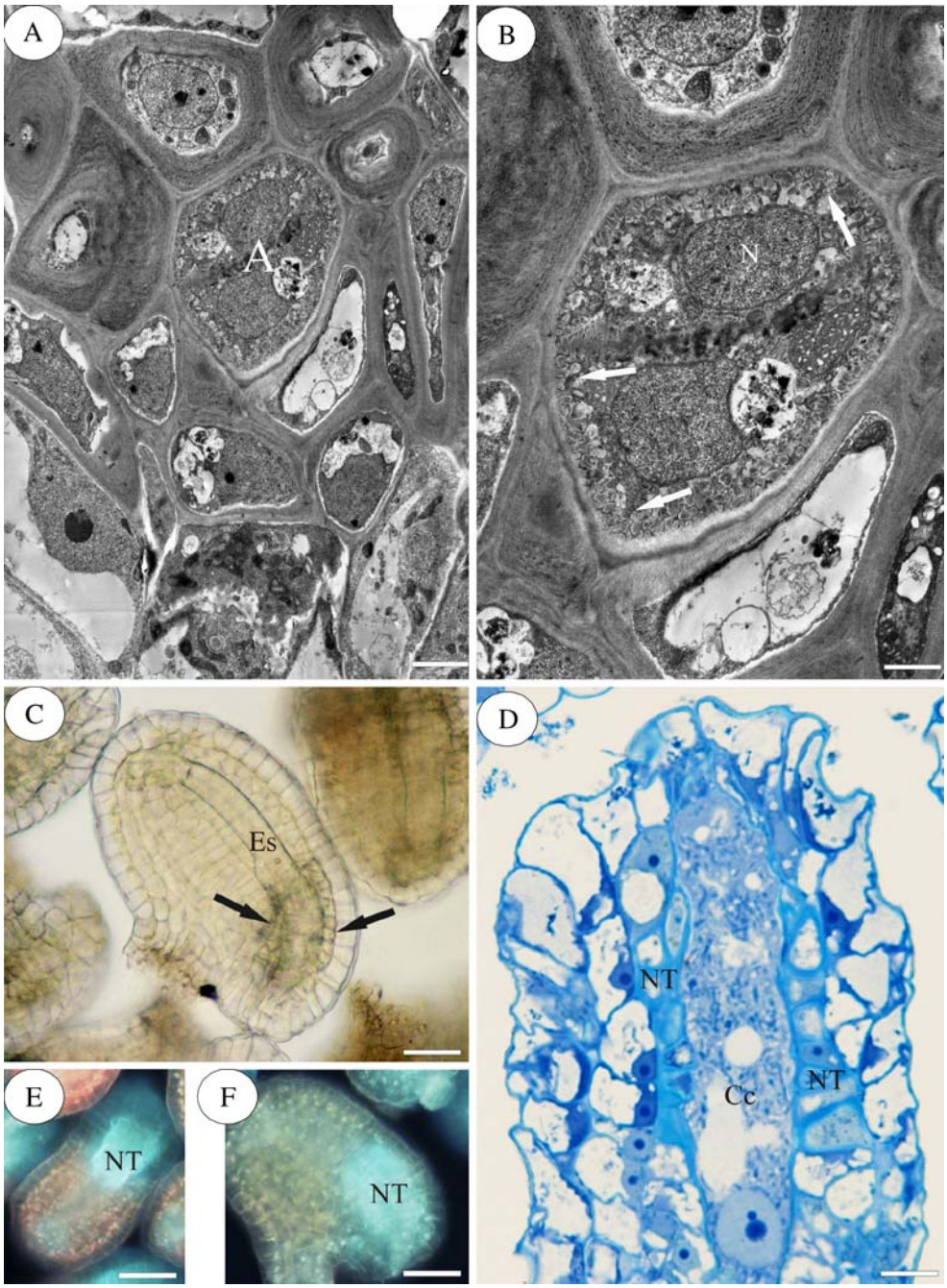

Fig. 3 Ultrastructure and anatomy of ovule of *Genlisea aurea*. Transverse sections of hypostase and antipodals (**a**), *bar*=2 µm. **b** transverse section of antipodals note prominent nucleus (*N*) and wall ingrowths (*arrows*), *bar*=0.8 µm. **c** Whole ovule showing curved embryo sac (*Es*) and micropylar nutritive tissue forming a jacket-like structure (*arrows*), *bar*=20 µm. **d** semithin section through ovule showing central cell (*Cc*) and nutritive cells with prominent thick cell wall (*NT*), *bar*=8 µm. **e** and **f** strong fluorescence of cell walls of nutritive cells (*NT*) under UV light, *bar*=43 and 31 µm

the base of the ovule. In the micropylar part of the ovule is a massive (several-layered) nutritive tissue of integumental origin, which forms a thick jacket around the embryo sac (Fig. 5c). The nutritive tissue cells have thicker walls than other integumental cells. Close to the central part of the embryo sac is a well-developed integumental tapetum (Fig. 5c). It consists of one layer of radially extended cells. At the chalazal pole of the ovule is a well-developed hypostase (Fig. 5c). Collapsed nucellar cells are distributed between the embryo sac and the integumental cells.

G. lobata

G. lobata forms a stalked spherical placenta covered with numerous (ca. 40) small ovules (Fig. 6a). The apex of the placenta is sterile and lacks ovules (not shown). The placenta consists mainly of highly vacuolated parenchymatic cells. The nucleus of the placental parenchymatic cells contains a protein inclusion. The placental cells possess plastids with starch (Fig. 6b). Intracellular spaces are well developed in the placenta and below the ovules (Fig. 6c). The ovule is

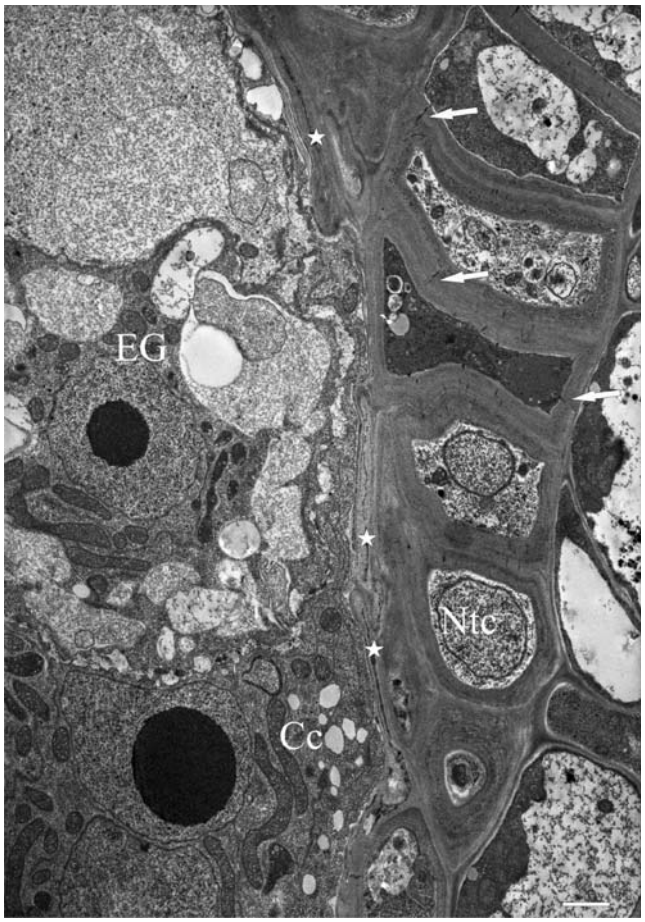

Fig. 4 Ultrastructure of nutritive tissue cells (*Ntc*) and mature embryo sac: egg cell (*EG*) and central cell (*Cc*). Note collapsed cells with remains of protoplast (*star*) between embryo sac and integumental cells and plasmodesmata (*arrow*) between integumental cells, *bar*=1.2 μm

tenuinucellate and unitegmic, ca. 130 μm long. The integument is partially fused with the funiculus, but the apex of the integument is free (Fig. 6c, d). The free part of the funiculus is prominent and well visible at the base of the ovule. In contrast to *G. aurea*, in *G. lobata* the apex of the integument is elongated and the micropyle adheres to the placental epidermis (Fig. 6c). The embryo sac is situated mainly in the middle and chalazal parts of the ovule (Fig. 6c). The embryo sac is curved, forming an arc. The chalazal part of the embryo sac is narrow, while the micropylar part is broad. The ES has contact, laterally and at the micropylar pole, with the integumental epidermis. Neither Heidenhain's hematoxylin and alcian blue (Fig. 6c) nor the PAS reaction revealed any special cells with very thick cell walls and dense cytoplasm near the ES. The epidermal cells of the chalazal pole of the ovule are large and prominent. The ovule may be classified as campylotropous.

One of the main aims of this work is to compare the structure of the ovule in *Genlisea* with that in *Utricularia* and *Pinguicula*. The ovule anatomy of *Utricularia* and *Pinguicula* (especially *Utricularia*) has been described in detail by various authors (Merz 1897; Merl 1915; Khan 1954, 1992; Płachno and Świątek 2008), so we concentrate here on their general morphology.

Pinguicula

In all examined species (Fig. 7b–d) the free part of the funiculus is well visible at the base of the ovule. The apex of the integument is elongated and the micropyle does not adhere to the funiculus.

Utricularia

The micropyle closely adheres to the raphe (Fig. 7f–h). The micropylar part of the integument borders the placenta (Fig. 7e). The embryo sac contacts the placenta (not shown).

Discussion

Ovules in Lentibulariaceae

Our work here suggests that *Genlisea* ovules are more similar to *Pinguicula* than to *Utricularia*. The free part of the funiculus is well visible in both *Pinguicula* and *Genlisea*. *Genlisea* ovules have one main character in common with *Utricularia* subgenus *Polypompholyx*: a well-developed funiculus. In the other subgenera of *Utricularia* the ovules are sessile. In *Utricularia* subgenus *Polypompholyx*, however, nutritive tissue makes the funiculus massive (Lang 1901; Merl 1915; Siddiqui 1978a; Płachno and Świątek 2008), unlike in *Genlisea* where nutritive tissue is absent from the funiculus.

There appears to be an evolutionary trend in the position of the micropyle in relation to the funiculus in Lentibulariaceae. In *Pinguicula* species and *G. lobata* the micropyle is separated from the funiculus. In *Genlisea* subgenus *Genlisea* the apex of the integument is curved so that the micropyle adheres to the funiculus. In *Utricularia* (subgen. *Bivalvaria* and *Utricularia*) the micropyle adheres to the funiculus. This tendency may be associated with the behavior of the embryo sac. As summarized by Farooq (1964, Table 1) the embryo sac remains inside the ovule in *Pinguicula* and *Genlisea*, but grows through the micropylar canal in *Utricularia*. Finally, the apex of the embryo sac is extra-ovular and has direct contact with the placenta. As shown (Płachno and Świątek 2008) this is a placental nutritive tissue, which consists of active cells and its position and structure is related to the phylogenetic position within the genus. As follows from Merl's (1915) and our results, the nutritive tissue in the *Genlisea* ovule is of

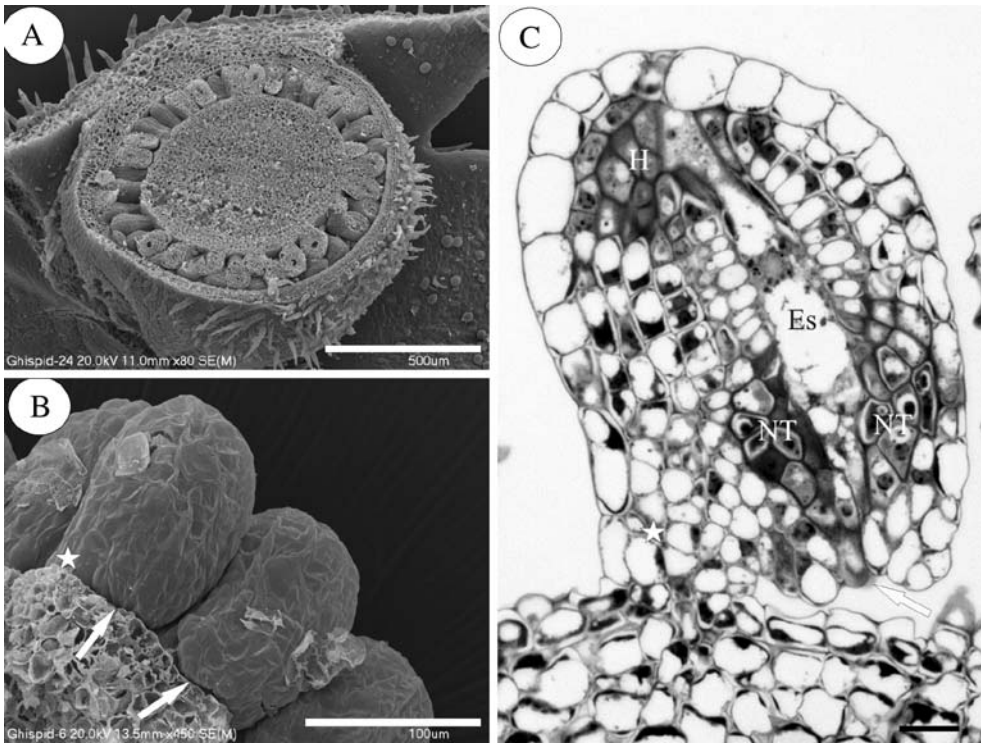

Fig. 5 Morphology and anatomy of ovule of *Genlisea hispidula*. **a** Transverse section of placenta with ovules, *bar*=500 μm. **b** morphology of ovules: *arrow* micropyle, *star* funiculus, *bar*=100 μm. **c** median longitudinal section of ovule: *H* hypostase, *arrow* micropyle, *star* funiculus, *Es* embryo sac, *bar*=16 μm

integumental origin. In *Utricularia* (sections Calpidisca and Oligocista and some other *Utricularia* species; Płachno and Świątek 2008, Płachno unpublished), the nutritive tissue consists of collenchymatous cells resembling those in *Genlisea*. Thus, the nutritive tissues in *Genlisea* and *Utricularia* are analogous because they arose from the different areas during the development of the ovule and placenta.

Within the genus *Genlisea*, Müller et al. (2006) and Fleischmann et al. (unpublished) consider subgenus *Tayloria* to be basal and subgenus *Genlisea* advanced. Alternatively, Płachno et al. (2007) inferred from the gland

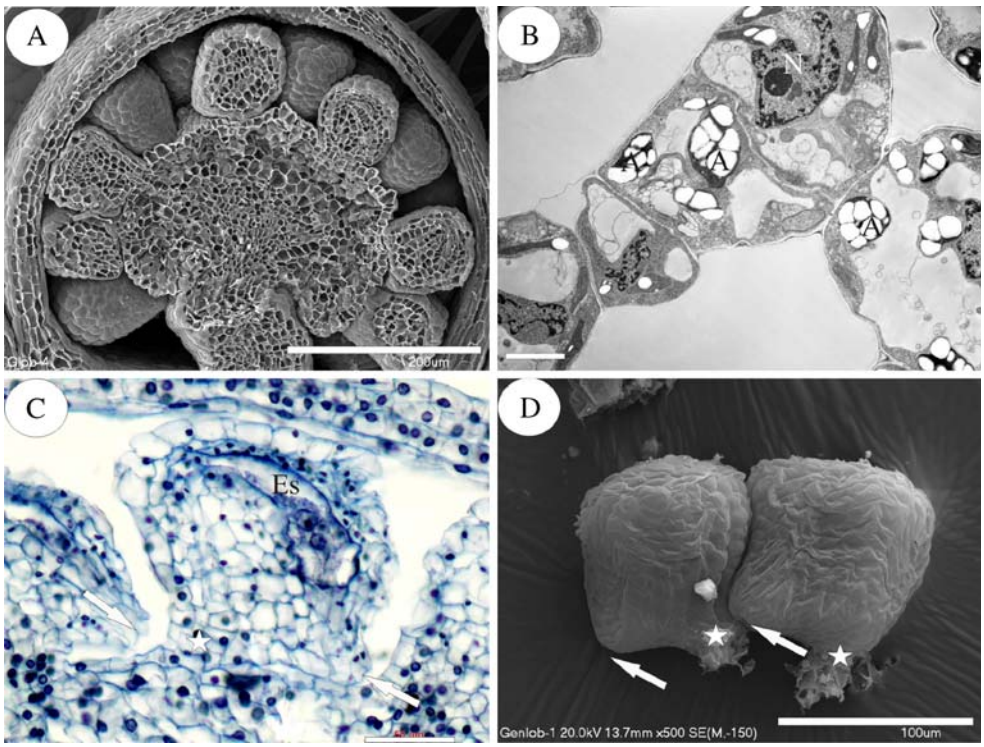

Fig. 6 Structure of placenta and ovule of *Genlisea lobata*. **a** Longitudinal section of placenta with ovules, *bar*=200 μm. **b** Placental parenchyma cells; *N* nucleus with protein crystals, *A* plastids with starch, *bar*=6 μm. **c** Median longitudinal section of ovule: *arrow* micropyle, *star* funiculus, *Es* embryo sac, *bar*=50 μm. **d** Morphology of ovules: *arrow* micropyle, *star* funiculus, *bar*=100 μm

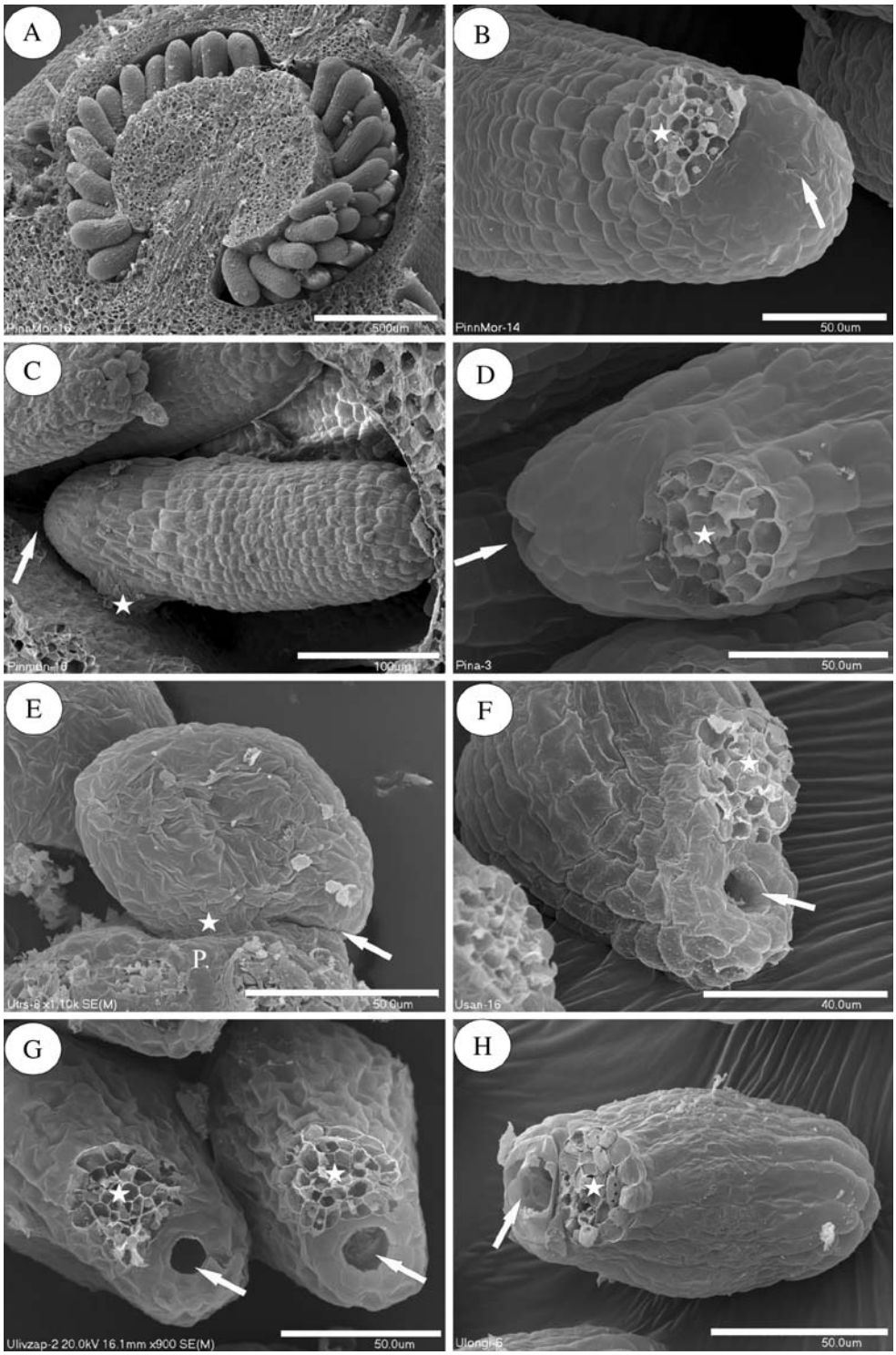

Fig. 7 Morphology of *Pinguicula* and *Utricularia* ovules. **a** Longitudinal section through the placenta with ovules of *P. moranensis*, *bar*=500 µm. **b** Ovule of *P. moranensis*: *arrow* micropyle, *star* funiculus, *bar*=50 µm. **c** Ovule of *P. moctezumae*: *arrow* micropyle, *star* funiculus, *bar*=100 µm. **d** Ovule of *P. agnata*: *arrow* micropyle, *star* funiculus, *bar*=50 µm. **e** and **f** *U. sandersonii*: *arrow* micropyle, *star* funiculus, *P.* placenta, *bar*=50 and 40 µm. **g** Ovules of *U. livida*: *arrow* micropyle, *star* funiculus, *bar*=50 µm. **h** Ovule of *U. longifolia*: *arrow* micropyle, *star* funiculus, *bar*=50 µm

distribution pattern in digestive chambers of *Genlisea* traps and from the findings of a molecular study by Jobson et al. (2004) that the concentration of hairs along the vascular bundles in species of subgenus *Genlisea* may be primitive. According to Jobson et al. (2004), active water pumping might have occurred in the *Genlisea* ancestor trap and the concentration of hairs along the vascular bundles could have helped in water transport. In recent *Genlisea* species, however, active transport of water with suspended organisms into traps has not been observed. In light of these hypotheses, ovule anatomy and morphology are especially interesting. The micropyle position in *G. lobata* (subgenus *Tayloria*) is similar to that in *Pinguicula* species. In *Pinguicula*, micropylar nutritive tissue as in *Utricularia* or

Genlisea does not occur, but an integumental tapetum is developed (Kopczyńska 1964; Farooq 1964). In *G. lobata* we found no micropylar nutritive tissue either. These results may suggest that species of subgenus *Tayloria* have more primitive characters, but further detailed studies are needed for confirmation.

According to Jobson and Albert (2002), in *Utricularia* the relative rates of nucleotide substitution in seven loci occurred four to 14 times faster than in *Pinguicula*. Also Müller et al. (2004, 2006) found the extreme DNA mutational rates in *Utricularia* and *Genlisea* in comparison to *Pinguicula* and moreover, that *Genlisea* and *Utricularia* exhibit substitutional rates which were the highest in the angiosperms. According to Müller et al. (2004, 2006) these changing in Lentibulariaceae genome well correspond to unusual nutritional specialization in *Genlisea* and *Utricularia* (so-called predictable prey capture hypothesis—Ellison and Gotelli 2009). Alternatively, unique molecular mutation (eg. in *cox*I subunit of cytochrome *c* oxidase) allowed to develop of morphological diversity and unique trap structures in *Genlisea* + *Utricularia* clad (Jobson and Albert 2002, Jobson et al. 2004, so-called energetics hypothesis—Ellison and Gotelli 2009). We also think that there is a positive connection between complication of the trap characters causing prey specialization and changes in Lentibulariaceae genome and moreover both hypotheses well complemented each other. However, as we show some generative characters (in case *Genlisea*) are more conservative than vegetative.

Special chalazal nutritive tissue or hypostase?

Several authors who studied the embryology of Lentibulariaceae have described the occurrence of a special chalazal nutritive tissue at the chalazal pole of the ovule in *Genlisea* (*G. aurea*; Merl 1915) and several species of *Utricularia* (e.g., Merz 1897; Lang 1901; Merl 1915; Khan 1954; Farooq 1964; Siddiqui 1978). In *U. aurea*, Khan (1954) described it as a group of cells which surround the base of the ES and are smaller than neighboring cells. In *Utricularia uliginosa* this tissue consists of thick-walled cells which are loosely arranged. At the first stage they have dense cytoplasm but later their contents are lost (Farooq 1965). According to Lang (1901), the chalazal nutritive tissue is well developed and prominent in *Utricularia multifida*. Merl (1915) noted nutritive tissue at the chalazal pole of the *G. aurea* ovule. Our results indicate that the special chalazal nutritive tissue in *Genlisea* and *Utricularia* is simply a hypostase. The position, anatomy and ultrastructure of this tissue closely correspond to the definition of the hypostase given by Batygina and Shamrov (1999). In his summary of the embryology of Lentibulariaceae, however, Khan (1992) briefly mentioned that a hypostase with lignified cell walls occurs in this family. He also mentioned chalazal nutritive tissue as another tissue type besides the hypostase in the ovule in this family. In disagreement with our results, according to Conran (1996), there is no hypostase in Lentibulariaceae.

Many authors consider that the principal function of the hypostase is to supply nutrients to gametophyte structures (e.g., Tilton 1980; Boesewinkel and Bouman 1984; Batygina and Shamrov 1999). In the case of *Genlisea*, the occurrence of elaborate wall labyrinths in the antipodals confirms that they are responsible for supplying nutrients from the hypostase to the central cell of the megagametophyte. Our ultrastructural study is in accordance with the work of Chamberlin et al. (1993): using autoradiographic techniques, they showed an accumulation of labeled assimilates in the integumentary tissue adjacent to the micropylar and chalazal poles of the embryo sac; they suggested that the chalazal vascular trace and two adfunicular vascular strands are the pathways for accumulation of the labeled assimilates in these regions of the ovule. In *Genlisea*, the funiculus does not have vascular tissue but is still the only way for metabolites to be transported from the placenta to the ovule tissue. This is unlike some *Utricularia* species (subgen. *Bivalvaria* and *Utricularia*) in which the embryo sac contacts the placental nutritive tissue, perhaps providing another pathway of metabolite transport directly from the placental nutritive tissue to the embryo sac (Płachno and Świątek 2008; Khan 1954, 1992).

Concluding remarks

We confirmed differences between the ovules of *Genlisea* subgenus *Genlisea* and subgenus *Tayloria*. Chromosome numbers also differ between these subgenera. Subgenus *Tayloria* contains species with low chromosome numbers; subgenus *Genlisea* contains high polyploids (Greilhuber et al. 2006; Płachno unpublished). Our knowledge of *Genlisea* cytology is still fragmented. Ovules in *Genlisea* retain characters in common with *Pinguicula* (ovules with free funiculus, ES remaining in the ovule), which were inherited from a common ancestor. However, in ovules of subgenus *Genlisea* the micropyle tends to be closer to the funiculus and an unusual jacket-like nutritive tissue of integumental origin is formed. The most specialized ovules in Lentibulariaceae evolved in the genus *Utricularia*. In this genus also the most sophisticated traps evolved within the family (Lloyd 1942; Juniper et al. 1989). Thus, in terms of both vegetative and generative characters the genus, *Utricularia* occupies an advanced position in the evolution in Lentibulariaceae.

Müller et al. (2006) showed that Madagascan and East African *Genlisea margaretae* has affinities to neotropical

Genlisea species but not to other African *Genlisea* species studied. The recent distribution of subgenus *Genlisea* could be interpreted to mean that the genus *Genlisea* developed before the breakup of Gondwana. Thus, not only the unique *Genlisea* trap but also *Genlisea* nutritive tissue are ancient inventions.

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Conflicts of interest statement The authors declare that they have no conflict of interest.

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