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Morphology, Biometry, and Taxonomy of Freshwater and Marine Interstitial *Cyphoderia* (Cercozoa: Euglyphida)

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ABSTRACT. Good taxonomy is essential for ecological, biogeographical, and evolutionary studies of any group of organisms. Therefore, we performed detailed light- and scanning electron microscopy investigations on the shell ultrastructure and biometric analyses of the morphometric variability of five freshwater and marine interstitial testate amoebae of the genus *Cyphoderia* (*C. trochus* var. *amphoralis*, *C. ampulla*, *C. margaritacea* var. *major*, *C. compressa*, and *C. littoralis*), isolated from different populations in Bulgaria and Switzerland. Our aims were (1) to clarify the morphological characteristics of these taxa, and (2) to compare the morphology of a given taxon (*C. ampulla*) among different locations in Bulgaria and Switzerland as a first step towards an assessment of the geographical variation within a supposedly cosmopolitan taxon. Four of the studied taxa are characterized by a well-expressed main-size class and by a small size range of all the characters and can be defined as size-monomorphic species. Based on these results, the following systematic changes are proposed: *C. major* (Penard, 1891) n. comb. (Syn.: *C. margaritacea* var. *major* (Penard, 1891) and *C. amphoralis* (Wailes & Penard, 1911) n. comb. (Syn.: *C. trochus* var. *amphoralis* (Wailes & Penard, 1911)). However, we also show significant morphological variability between the Swiss and Bulgarian populations of *C. ampulla*, suggesting the possible existence of more than one taxon within this species. Further studies are required to assess (1) if these two morphologically different taxa represent individual species, (2) if so, if more species exist, and if this diversity is due to limited distribution ranges (endemism) or if several closely related taxa occur together in different geographical areas.

Key Words. Biometry, *Cyphoderia*, *Cyphoderia amphoralis* n. comb., *Cyphoderia major* n. comb., morphology, taxonomy, testate amoebae.

RELIABLE taxonomy is an essential prerequisite for studies of the ecology, biogeography, and evolution of any group of organisms. Unfortunately, poor taxonomy is one of the curses of the study of free-living protists, leading to endless debates about the existence or lack of endemism (Mitchell and Meisterfeld 2005). This problem is partly due to the difficulty in defining what a species is for organisms that reproduce asexually or by inbreeding (Schlegel and Meisterfeld 2003), although these biological features are not unique to protists and should not be used as an excuse for not improving the taxonomy (Finlay 2004).

Among the different groups of protists, the polyphyletic groups of testate amoebae are good models for taxonomy and evolutionary studies because of their diversity, ubiquity, the presence of a shell which is taxonomically diagnostic, and their long (but still very poorly studied) fossil record (Porter and Knoll 2000). Furthermore, the shell or test that they produce is preserved in peat and sediments making them useful in palaeoecology (Charman 2001). The range of current and potential uses of testate amoebae in applied ecology (e.g. biomonitoring) (Foissner 1997, 1999) and palaeoecology makes it even more important to improve the taxonomy of this group (Mitchell, Charman, and Warner 2008).

On the one hand, some testate amoebae have been shown to display considerable shell polymorphism (Chardez 1989; Bobrov, Yazvenko, and Warner 1995; Lahr, Bergmann and Lopes 2008) or phenotypic plasticity (Wanner 1999; Wanner, Esser, and Meisterfeld 1994; Wanner and Meisterfeld 1994). On the other hand, relatively small morphological differences, used to define infrageneric taxa, have been shown to correspond to significant differences in the small subunit rRNA gene (Lara et al. 2008). Indeed, all morphologically distinct testate amoebae, such as Arcellinida and Euglyphida, for which a DNA sequence has been obtained to this date have also proven to be distinct from a molecular standpoint (Lara et al. 2007, 2008). Nevertheless, one of the most serious taxonomic problems of testate amoebae is the existence of many poorly defined infraspecific taxa (i.e. subspecies, varieties, and forms). It is both imperative to study these taxa in detail and to revise their status. Our focus here is on *Cyphoderia*, a genus of euglyphid testate amoeba.

The genus *Cyphoderia* Schlumberger, 1845, with the type species *Cyphoderia ampulla* (Ehrenberg, 1840) Leidy 1879, is one of the first testate amoeba genera to be described. *Cyphoderia* species live mainly in freshwater and moss but are also found in marine interstitial habitats. Since its description more than 20 taxa have been assigned to the genus (Chardez 1991). Some of these species have a very distinctive morphology as well as precise ecological requirements: *Cyphoderia calceolus*, *Cyphoderia trochus*, and *Cyphoderia myosurus* are specialized deep-water species, *Cyphoderia compressa* and *Cyphoderia littoralis* are marine psammobiotic species (Golemansky 1973, 1979; Penard 1902; Wailes and Penard 1911). These last two species were never mentioned from freshwater environments. Besides, there are many other taxa, mainly infrasubspecific taxa—varieties, which are poorly described, without detailed morphometry or microphotographs (e.g. *C. ampulla* var. *thomasi* Chardez, 1956; *C. ampulla* var. *bicornis* Stepanek, 1963; *C. littoralis* var. *shimodensis* Sudzuki, 1979, etc.). Most of them have no taxonomical value and fall into the range of natural variability of a nominal species or represent teratological forms of the same species. In support of this opinion is the fact that many of the earlier researchers observed numerous transitions between different morphs and mentioned polymorphism in *C. ampulla* (Leidy 1879; Penard 1902; Wailes and Penard 1911). Such observations led Leidy (1879) to lump all observed taxa into the type species *C. ampulla*.

During our investigations of the freshwater and marine testate amoebae of Bulgaria and Switzerland we observed several taxa of the genus *Cyphoderia*. We performed detailed light- and scanning electron microscopy (SEM) investigations on the shell ultrastructure and biometric analysis of the morphometric variability in these species. Our aims were (1) to do a comparative analysis

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Table **1.** List of the *Cyphoderia* studied and sampling locations.

Previous name	Taxon	Sampling location	Sampling date	Co-ordinates		Altitude (m)
C. trochus var. *amphoralis*	*C. amphoralis*	Rila (BG)	August 2005	41°59′N	24°10′E	1,109
C. ampulla	*C. ampulla*	Moiry (CH)	July 2006	46°08′N	07°34′E	2,310
C. ampulla	*C. ampulla*	Rhodopes (BG)	July 2005	41°59′N	24°10′E	1,109
C. ampulla	*C. ampulla*	Rila (BG)	August 2005	42°12′N	23°22′E	1,960
C. ampulla	*C. ampulla*	Vitosha (BG)	August 2006	42°36′N	23°17′E	1,850
C. ampulla var. *major*	*C. major*	Rila (BG)	August 2005	42°12′N	23°22′E	1,960
C. compressa	*C. compressa*	Black Sea (BG)	July 2006	43°10′N	27°56′E	0
C. littoralis	*C. littoralis*	Black Sea (BG)	July 2006	43°10′N	27°56′E	0

BG, Bulgaria; CH, Switzerland.

of morphologically different taxa in order to clarify their morphological characteristics, and (2) to compare the morphology of a given taxon (*C. ampulla*) among different locations in Bulgaria and Switzerland as a first step towards an assessment of the geographical variation within a supposedly cosmopolitan taxon.

MATERIALS AND METHODS

Sampling and morphological analyses. A total of eight *Cyphoderia* populations was sampled from five different localities in Bulgaria and Switzerland from July 2005 to August 2006 (Table 1). The salinity of the underground water at the sampling sites of *C. compressa* and *C. littoralis* ranged between 16‰ and 19‰. For each freshwater and marine population several morphological characteristics were measured: shell length, shell height, and shell width, and long and short axes of the pseudostome (aperture) were analysed with an ''Amplival'' (Zeiss-Jena, Germany) microscope under 400X magnification (Fig. 1). Specimens were transferred in a drop of glycerol in order to maintain the specimens in a given position during the measurements. One hundred specimens per population were analysed. For the *C. ampulla* population from Moiry (Switzerland) only 50 specimens were analysed owing to the low number of specimens available in the sample and all measurements for this population were performed on SEM images.

To determine the dimensions and degree of overlapping of scales and the number of scales surrounding the pseudostome, 10 specimens from each population were observed by SEM. For this, specimens were isolated using a glass micropipette, washed several times by transfers in distilled water, and then individual

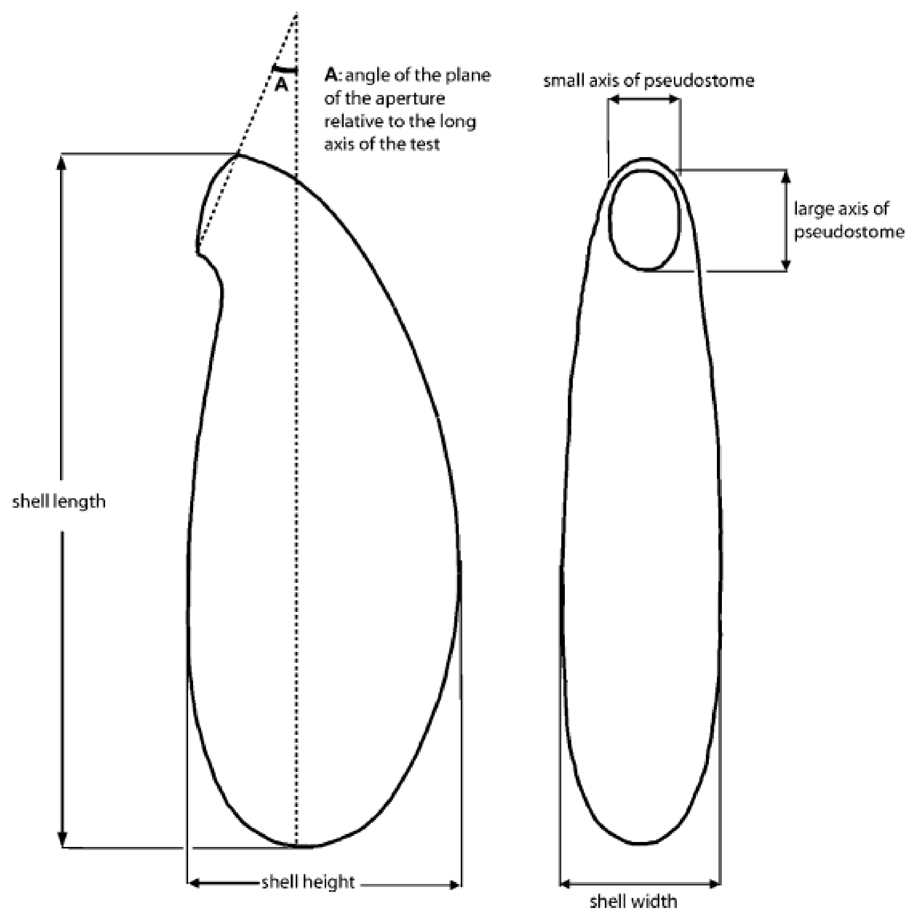

Fig. **1.** Schematic outline of the testate amoeba *Cyphoderia* showing different morphological characteristics measured.

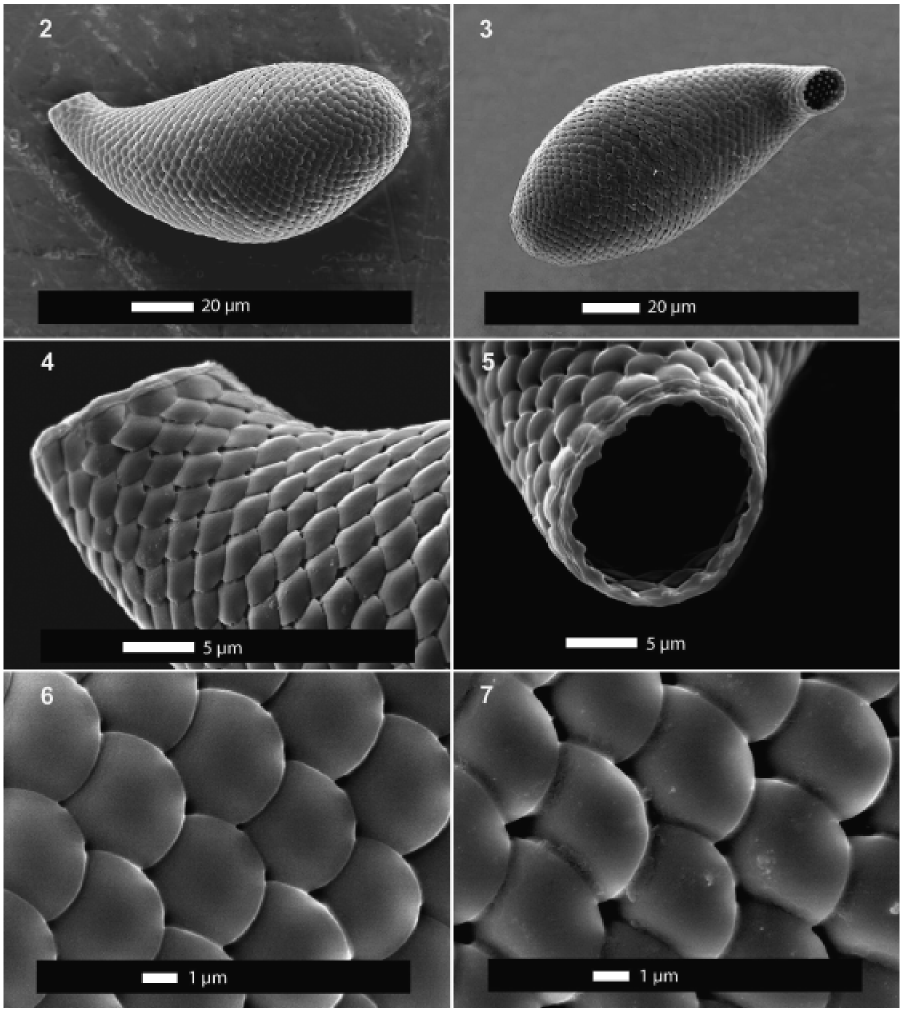

Fig. **2–7.** Scanning electron micrographs of *Cyphoderia amphoralis* from Rila (Bulgaria). **2, 3.** Shells; **4, 5.** Detail of the pseudostome; **6, 7.** Detail of the scales arrangement.

shells were positioned with a single-hair brush either onto a small drop of Araldite on an aluminium stub or directly on an aluminium stub. The preparations were then air dried in a desiccator for 1 wk. The shells were coated with gold in vacuum coating unit. Specimens were observed either with a JEOL JSM-5510 microscope (Tokyo, Japan) at 10 kV or with a Philips XL30 FEG microscope (Amsterdam, The Netherlands) at 3 kV.

Numerical analyses. For each morphometric variable, we first calculated basic statistics: arithmetic mean; median (M); standard deviation (SD); standard error of mean (SE); coefficient of variation in % (CV); and extreme values (min and max). We then performed principal component analyses (PCA) in order to assess the range of morphological variation within and among populations and species and determine the discriminating value of the different morphological variables. Three semi-quantitative variables were used in the PCA to characterize the overlapping of the scales: (1) no overlap of scales, (2) low degree of overlap of scales; and (3) high degree of overlap of scales. We compared the length and the width of *C. ampulla* populations from Rhodopes, Rila, and Vitosha with a Kruskal–Wallis non-parametric test using Nemenyi post hoc multiple comparisons (Zar 1999). Statistical analyses were performed using the computer program STATISTICA, version 7.0 and with the R statistical software (R Development Core Team 2005).

RESULTS

Morphological descriptions. A total of 750 *Cyphoderia* specimens belonging to eight different populations were analysed. Owing to important morphological differences between *C. ampulla* population from Switzerland (Moiry) and from Bulgaria (Rila, Rhodopes, and Vitoscha), these morphotypes are presented separately.

***Cyphoderia amphoralis* (Wailes & Penard, 1911)** n. comb. (Fig. 2–7, 36).

(Syn.: *C. trochus* var. *amphoralis* Wailes & Penard, 1911)

Material examined. The specimens were isolated from the samples of wet *Sphagnum* mosses in the vicinity of ''Maljovitsa'' hut, Rila Mountains, Bulgaria (Table 1).

Description. The shell is yellowish–brownish in colour, transparent, and retort-shaped laterally. In ventral and dorsal views it is elongated–elliptical in outline, with rounded posterior end and tapering to the anterior end (Fig. 2, 3). The cross section of the main test body is circular and the shell reaches its maximum diameter at about 1/3 from its posterior end. In lateral view the shell is curved towards the pseudostome, forming a well-expressed, slightly laterally compressed neck. The pseudostome is oval, oblique, and surrounded by about 16–18 apertural scales. The rim around the

Table **2.** Comparative morphometrical characters of the *Cyphoderia* populations studied.

Taxon	Sampling location	Shell colour	Cross section	Form of scales	Dimensions of scales (µm)	Number of scales surrounding the aperture	Imbrication of scales
C. amphoralis	Rila (BG)	Yellowish-brownish	Circular	Circular, biconvex	2.5–6.0	16–18	Impressive
C. ampulla	Rhodopes (BG)	Colourless or yellowish	Circular	Circular, feebly biconvex	2.0–3.5	18–20	Absent or feebly imbricated
C. ampulla	Rila (BG)	Colourless or yellowish	Circular	Circular, feebly biconvex	2.0–3.5	18–22	Absent or feebly imbricated
C. ampulla	Vitosha (BG)	Colourless or yellowish	Circular	Circular, feebly biconvex	2.5–4.0	18–22	Absent or feebly imbricated
C. ampulla	Moiry (CH)	Colourless or yellowish	Circular	Circular or oval, plane	1.8–2.7 × 1.4–1.9	25–33	Non-imbricated
C. major	Rila (BG)	Colourless or yellowish	Circular	Circular or oval, plane	1.6–2.1	35–40	Non-imbricated
C. compressa	Black Sea (BG)	Colourless	Oval	Kidney-shaped, plane	2.2–2.5 × 1.7–2.0	22–26	Non-imbricated
C. littoralis	Black Sea (BG)	Colourless	Circular	Circular, feebly convex	1.7–2.0	18–22	Imbricated

BG, Bulgaria; CH, Switzerland.

pseudostome is covered by a thin organic cement (Fig. 4, 5). The angle of the plane of the pseudostome relative to the long axis of the test is variable and ranges from 1° to 35°. The shell is composed of circular, biconvex, impressively imbricated scales (see Table S1), which differ in size depending on their position on the shell: where the shell reaches largest diameter the scales are 2 or 3 times larger than those located in the pseudostome and aboral regions (5–6 µm and 2.5–3 µm, respectively). The arrangement of scales is also characteristic: they insert obliquely relative to the shell surface and there are clearly imbricated (Fig. 6, 7). This arrangement of scales gives a characteristic indented and rougher outline to the shell of *C. amphoralis*.

Measurements (in µm). (See Table 2 and Table S1)

Biometry. Shell dimensions are rather constant with coefficients of variation of all measured characters lower than 5% (see Table S1). The most stable characters in the studied population are shell length (coefficient of variation [CV]: 3.70%), shell width (CV: 4.04%), and the large axis of the pseudostome (CV: 4.0%). Our values for all characters are slightly higher than those given by Wailes and Penard (1911). The analysis of the size frequency distribution indicates that *C. amphoralis* is a size-monomorphic species, characterized by a comparatively small size range (see Table S1). For example, the shell length of all individuals measured ranges from 139 to 170 µm and about 85% of them have a shell length of 140–155 µm. The frequency analysis of the shell diameter and of the large axis of the pseudostome shows that in all specimens measured these characters are very constant and range in close limits, 56–69 µm and 17–20 µm, respectively.

***Cyphoderia ampulla* (Ehrenberg, 1840) Leidy, 1879** (Fig. 8–13, 36)

Material examined. The specimens were isolated from samples of wet *Sphagnum* mosses at the following three localities in Bulgaria: ''Maljovitsa'' hut, Rila Mountains; reservoir ''Batak'', Rhodopes Mountains; and ''Platoto'', Vitosha Mountain (Table 1).

Description. The shell is colourless or yellowish, transparent, and retort-shaped (Fig. 8, 9). The cross section is circular. In lateral view the shell is elongated, rounded at the posterior end and is curved towards the pseudostome, forming a well-expressed neck, terminating with a circular or rarely slightly oval, oblique pseudostome. The pseudostome is surrounded by about 18–22 apertural scales and has a rim with a thin covering of organic cement (Fig. 10, 11). The angle of the plane of the pseudostome relative to the long axis of the test ranges from 10° to 30°. The shell is composed of numerous circular, feebly biconvex, comparatively small (2.0–3.5 µm) siliceous scales, which are slightly imbricated and almost equal in size throughout the shell. The scales are regularly arranged in diagonal rows, about 16–18 in the mid-region of the shell, and are held together by an abundant cement, which frequently covers them, giving a smooth outline and hexagonal appearance of the scale (Fig. 12, 13). Based on the SEM study of the shell's ultrastructure and on the regularity of this character in all studied specimens we supposed that this cement is organic.

Measurements (in µm). (See Table 2 and Table S1)

Biometry. The coefficients of variation for all characters are comparatively low, between 3.08 and 6.46 (see Table S1), but they differ among populations. The specimens from the Rila Mountains are more variable than those from the Vitosha and Rodopes Mountains (5.08–6.46%, 3.08–5.75%, and 3.24–4.24%, respectively).

The analysis of the size frequency distribution indicates that *C. ampulla* is a size-polymorphic species with respect to shell length and shell diam. (see Table S1). The shell lengths from Vitosha, Rila, and Rhodopes populations range, respectively, between 115 and 142, 112 and 150, and 107 and 129 µm. The shell width of Vitosha and Rila populations is also variable and without a well-expressed main size-class: 45–55 µm and 42–60 µm, respectively. The population of the Rhodopes is monomorphic with respect to this character: all individuals measured range between 40 and 47 µm. The large axis of the pseudostome is the most stable character in all populations: in more than 90% of the specimens in each population the diameter of the pseudostome ranges between 15 and 17 µm.

***Cyphoderia ampulla* (Ehrenberg, 1840) Leidy, 1879** (Fig. 14–19)

Material examined. The specimens were obtained from aquatic mosses collected in Moiry, Val d'Anniviers, Switzerland (Table 1).

Description. The general shape morphology and the color of these Swiss specimens of *C. ampulla* (Fig. 14, 15) are similar to those from Bulgaria described above. However, different ultrastructural features clearly distinguish the specimens of this

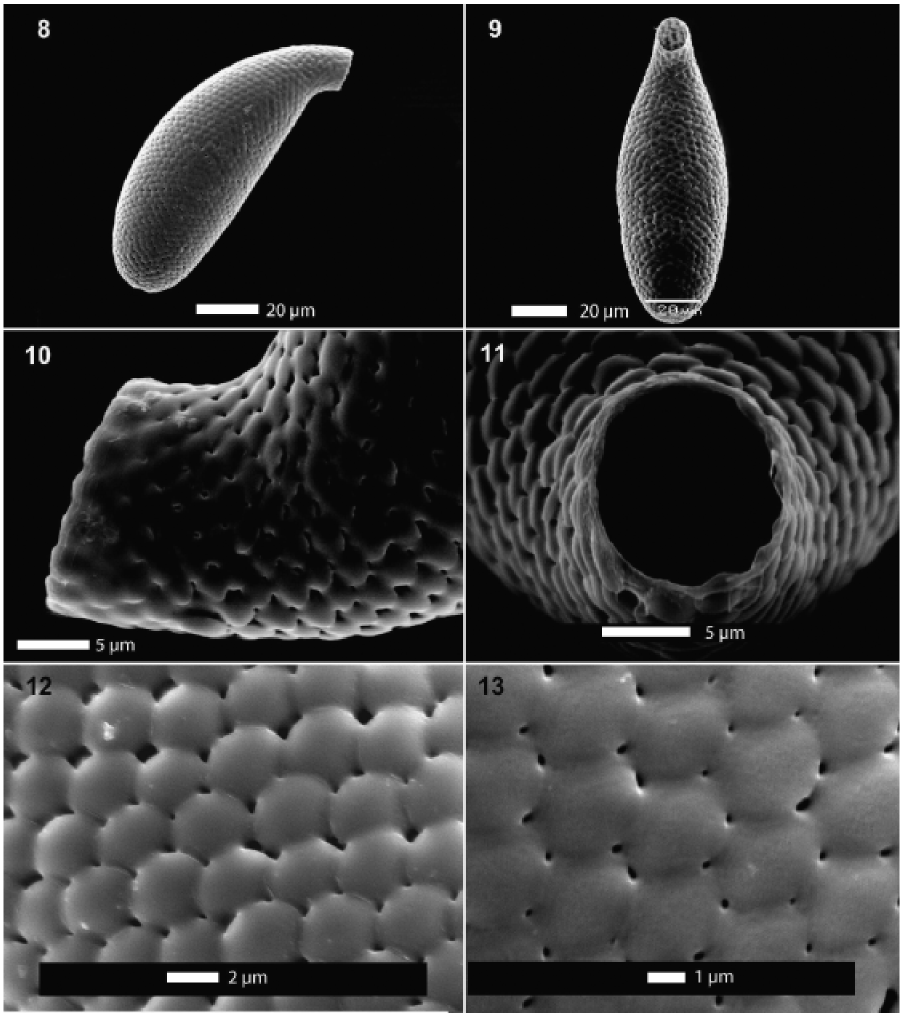

Fig. **8–13.** Scanning electron micrographs of *Cyphoderia ampulla* from Vitosha (Bulgaria. **8, 9.** Shells; **10, 11.** Detail of the pseudostome; **12, 13.** Detail of the scales arrangement.

population: (1) the pseudostome is surrounded by a higher number of apertural scales (25–33 vs. 18–22) (Fig. 16, 17); (2) the shell is composed of plane circular or oval siliceous scales (1.8–2.7 × 1.4–1.9 µm) vs. circular and feebly biconvex scales (Table 2); (3) the scales do not overlap (Fig. 4, 18, 19) there is no abundant organic cement covering the scales (Fig. 18, 19).

Measurements (in µm). (See Table 2 and Table S1)

Biometry. The shell length and width range, respectively, between 100 and 124 µm and between 38 and 46 µm. The morphometric dimensions of these specimens are usually smaller than the Bulgarian specimens (see Table S1).

***Cyphoderia major* (Penard, 1891**) n. comb. (Fig. 20–25, 36) (Syn.: *C. margaritacea* var. *major* Penard, 1891)

Material examined. The specimens were isolated from samples of wet *Sphagnum* mosses collected in the vicinity of ''Maljovitsa'' hut, Rila Mountains, Bulgaria (Table 2).

Description. The shell is large, colourless or yellowish, transparent, retort-shaped laterally and elongated–elliptical in outline in ventral and in dorsal views, with a maximum diameter in the mid-region of the shell (Fig. 20–22). The cross section is circular. In lateral view the shell is broadly rounded at the posterior end and curved at the anterior end towards the pseudostome, forming an elongated neck. The pseudostome is circular and oblique, is surrounded by about 35–40 apertural scales with clearly visible (SEM) denticles, and has a rim with a thin covering of organic cement (Fig. 23). The angle of the pseudostome plane relative to the long axis of the test ranges from 10° to 25°. The shell is composed of numerous small (1.6–2.1 µm), circular or oval, plane, comparatively thick (1.5–2.0 µm), and non-imbricated scales, which are regularly arranged side by side in diagonal rows about 34–37 in the mid-region of the shell (Fig. 24, 25). Each scale is surrounded by a ring of six perforations in the organic cement, which give a characteristic appearance of the shell of *C. major* when it is observed under the light microscope.

Measurements (in µm). (See Table 2 and Table S1)

Biometry. The coefficients of variation of all measured characters are low, between 3.36% and 4.49%: minimal variability was observed for shell diameter (3.36%) and the diameter of the pseudostome (3.68%), while shell length (4.15%) and ratio of length/diam. (4.49%) were slightly more variable. Our values correspond well to those given by Penard (1891; 1902) and Wailes and Penard (1911).

The size frequency distribution analysis indicates that *C. major* is a size-monomorphic species, characterized by a main-size class and a small size range (see Table S1). For example, 95% of all

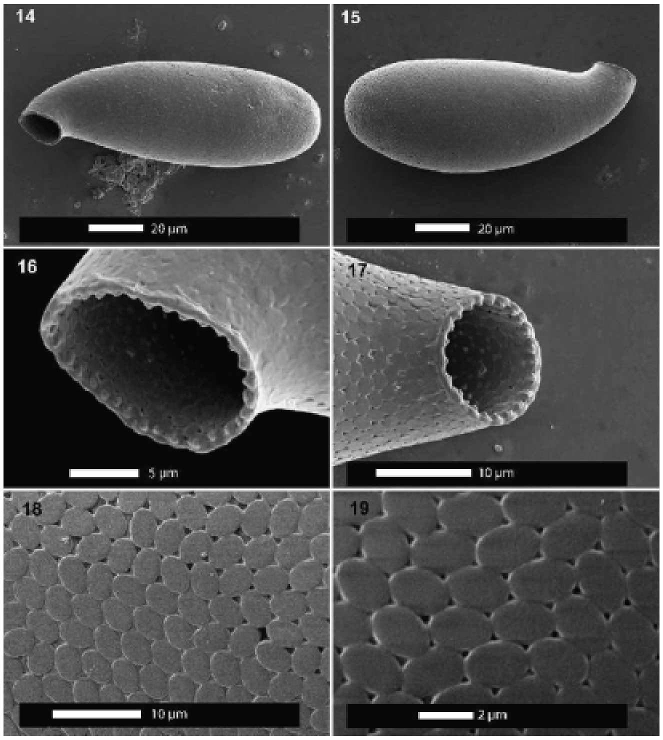

Fig. **14–19.** Scanning electron micrographs of *Cyphoderia ampulla* from Moiry (Switzerland. **14, 15.** Shells; **16, 17.** Detail of the pseudostome; **18, 19.** Detail of the scales arrangement.

observed specimens have a shell length ranging between 175 and 205 µm and only 5% have a shell length exceeding 205 µm. The variability of shell diameter is slightly higher but still the width of about 85% of all specimens range between 75 and 81 µm. The axis of the pseudostome in all the specimens measured ranges within close limits, 23–28 µm (see Table S1).

***Cyphoderia compressa* Golemansky, 1979** (Fig. 26–31)

Material examined. The specimens were isolated from a sample collected from a sandy beach, 6 m above the high-water mark gathered at the ''Galata'' Beach, Bulgarian Black Sea Coast (Table 1).

Description. The shell is colourless or yellowish, transparent, retort-shaped, laterally compressed, and reaches its maximum height at 1/4–1/3 from its posterior end (Fig. 26, 27). In cross section the shell is markedly oval, higher than wide. In lateral view the shell is curved towards the pseudostome, forming an elongated neck. The pseudostome is oblique and oval and the apertural rim has a thin covering of organic cement (Fig. 28, 29). The angle of the plane of the pseudostome relative to the long axis of the test is variable and ranges from 0° to $\sim 30°$. In ventral and dorsal views, the sides of the shell are almost parallel along most of the shell length. The shell is covered with characteristic kidney-shaped, plane scales (2.2–2.5 µm wide × 1.7–2.0 µm long). The scales are without imbrication and regularly arranged side by side on abundant organic cement (Fig. 30, 31). The organic cement is often visible in the relatively large interstices between scales and does not have any defined structure (Fig. 30).

Measurements (in µm). (See Table 2 and Table S1)

Biometry. The shell dimensions are relatively constant with coefficients of variation of all characters measured lower than 6% (see Table S1). The most stable characters in the Black Sea population are shell length (2.96%) and the shell length/width ratio (2.98%). Comparatively more variable are shell height (4.96%) and the shell width/height ratio (5.8%). Our values correspond well to those of Golemansky (1979), but it should be noted that in the latter's description the values for the shell width and height are inverted (largeur = height and epaisseur = width).

The analysis of the size frequency distribution indicates that *C. compressa* is a size-monomorphic species, characterized by a comparatively small size range (see Table S1). For example, the shell length of all the individuals measured ranged from 97 to 111 µm. Moreover, about 75% of them have a shell length of 101–107 µm. The frequency analysis of the shell width and of the large axis of pseudostome show that in all specimens measured these characters are very constant, 23–28 µm and 13–16 µm, respectively.

***Cyphoderia littoralis* Golemansky, 1973** (Fig. 32–35)

Material examined. The specimens were isolated from a sample collected from a sandy beach, 6 m above the high-water mark gathered at ''Galata'' Beach, Bulgarian Black Sea Coast (Table 1).

Description. The shell is colourless, transparent, and retort-shaped with a characteristic enlargement in the mid-region and tapering equally to the pseudostome and aboral extremity (Fig. 32–34). The cross section is circular. In lateral view the shell is curved at the anterior end, and forms a short neck, terminated with a slightly oval, oblique pseudostome. The rim of the pseudostome has a thin covering of organic cement (Fig. 33). The angle of the plane of the aperture relative to the long axis of the test ranges from 5° to $\sim 25°$. The shell is composed of numerous small (1.7–2.0 µm), circular, feebly convex scales, which overlap each other and are arranged in diagonal rows (Fig. 35). The organic cement is usually not seen between the scales.

Measurements (in µm). (See Table 2 and Table S1)

Biometry. The coefficients of variation of all measured characters are low—between 3.81% and 6.22%. Minimal variability was observed for shell length (3.81%), length/diam. ratio (3.84%), and shell diam. (4.02%). Slightly more variable are the large and small axis of pseudostome (5.10% and 6.22%, respectively). Our values correspond well to the original description of the species (Golemansky 1973) as well as data from Golemansky and Todorov (2004).

The size frequency distribution analysis indicates that *C. littoralis* is a size-monomorphic species, characterized by a main-size class with a small size range (see Table S1). For example, 97% of all specimens measured have a shell length ranging between 46 and 53 µm and only 3% have a shell length exceeding 53 µm. The shell width and the large axis of the pseudostome in all specimens measured are very constant and range within close limits, 22–27 µm and 10–12 µm, respectively (see Table S1).

Numerical analysis of the eight *Cyphoderia* populations. The PCA of the morphometric data shows that each population studied forms a distinct cluster with the exception of the *C. ampulla* populations from Batak, Rila, and Vitoscha, which are grouped together (Fig. 37). Shell dimensions variables, such as width, height, length, small axis of the pseudostome, and large axis of the pseudostome, are correlated with the first axis, while the degree of overlapping of plates, the width/height ratio, and the length/width ratio are mainly correlated with the second axis (data not shown). In this analysis, *C. major* clearly stands out by its large shell dimensions and *C. compressa* by its low W/H ratio and high L/W ratio (compressed shell). *Cyphoderia littoralis* is characterised by small shell size and overlapping scales, and *C. amphoralis* by the high degree of overlap of scales (Fig. 6, 7). The degree of overlap of scales further distinguishes two groups within

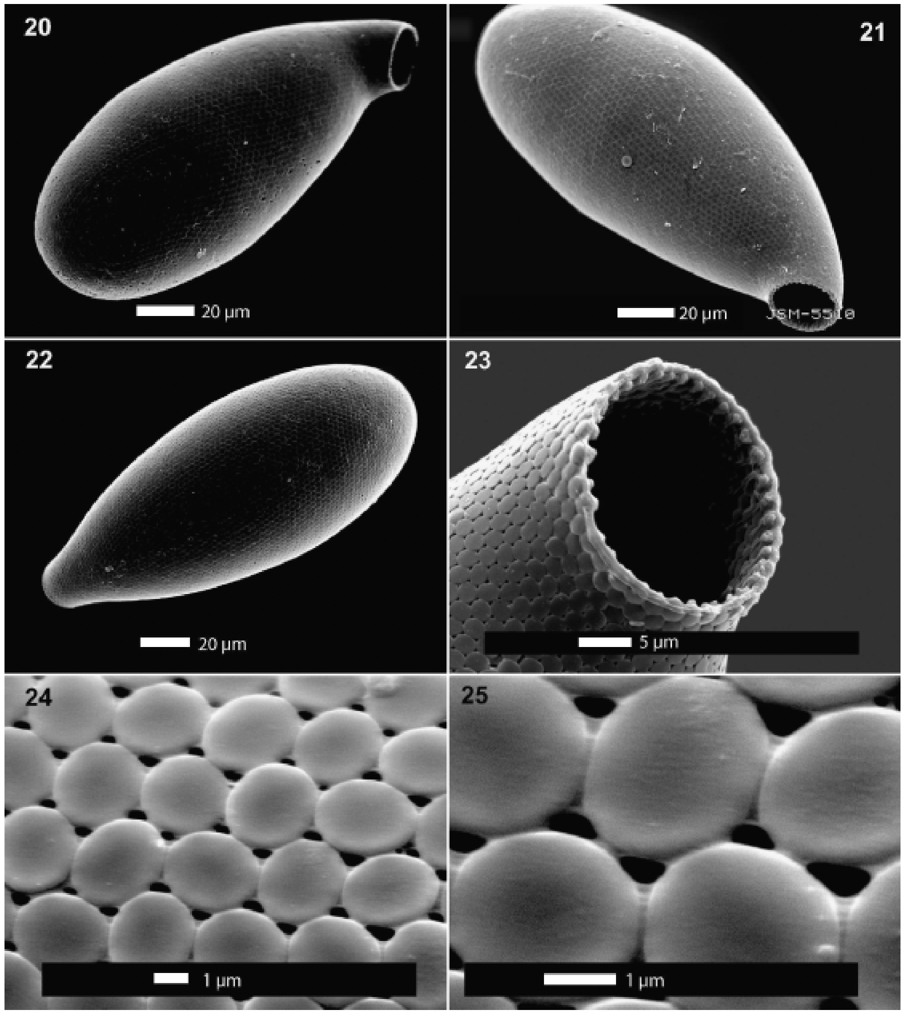

Fig. **20–25.** Scanning electron micrographs of *Cyphoderia major* from Rila (Bulgaria). **20–22.** Shells; **23.** Detail of the pseudostome; **24, 25.** Detail of the scales arrangement.

C. ampulla. In the first group, which includes all specimens from Moiry (Switzerland), the scales do not overlap at all (Fig. 18, 19). In contrast, in the second group, which includes specimens from Bulgaria (Rhodopes, Rila and Vitoscha), the scales overlap slightly (Fig. 12, 13).

A second PCA analysis (Fig. 38) of only the *C. ampulla* populations and four different morphological characters relevant for this taxon (i.e. degree of overlap of the scales, length, width, and length of the axis of the pseudostome) confirms the clear distinction between the Swiss and Bulgarian populations. Within the Bulgarian populations, the relative positions of specimens from Rhodopes are mainly concentrated on the left side of the cluster, owing to the smaller length and width dimensions of Rhodopes specimens as compared to specimens from Rila and Vitosha (Fig. 38).

Univariate analysis based on shell lengths and widths of *C. ampulla* from Rhodopes, Rila, and Vitosha further illustrates this. Indeed, significant differences in shell length and width were observed between the population of the Rhodopes and the other two populations (Rila and Vitosha), ($P < 0.05$; Kruskal–Wallis test unsing Nemenyi post hoc multiple comparisons, $N = 100$, Fig. 39, 40) but no significant differences were observed between the Rila and Vitosha populations ($P > 0.05$; Kruskal–Wallis test unsing Nemenyi post hoc multiple comparisons, $N = 100$, Fig. 39, 40).

DISCUSSION

Taxonomy of the main morphotypes. The classification of *Cyphoderia* species is mainly based on the shape and arrangement of the scales (i.e. imbricated or non-imbricated), as well as on the cross section of the shell (Chardez 1991). The shape of the scales caused some confusion in early studies of this genus: several authors described a hexagonal pattern in the shell structure of *C. ampulla* and concluded that the shell was built with hexagonal plates (Leidy 1879; Rhumbler 1896; Wailes and Penard 1911). However, Penard (1902) conducted shell disintegration experiments using acid and proved that the structural elements of the shell in *Cyphoderia* are not hexagonal but circular. Penard (1902) further pointed out that the hexagonal appearance may be due to the presence of cement material attached to the sites where the disks meet.

Based on the arrangement of these disks Wailes and Penard (1911) noted that in *C. ampulla* there are three groups of shells: (1) shells in which the disks touch one another, and appear circular, leaving transparent angular interstices; (2) shells in which the disks have a hexagonal appearance; and (3) shells in which the disks are separated from each other by an appreciable distance. In the same work Wailes and Penard made an attempt to classify all known species and varieties of *Cyphoderia* into three main types:

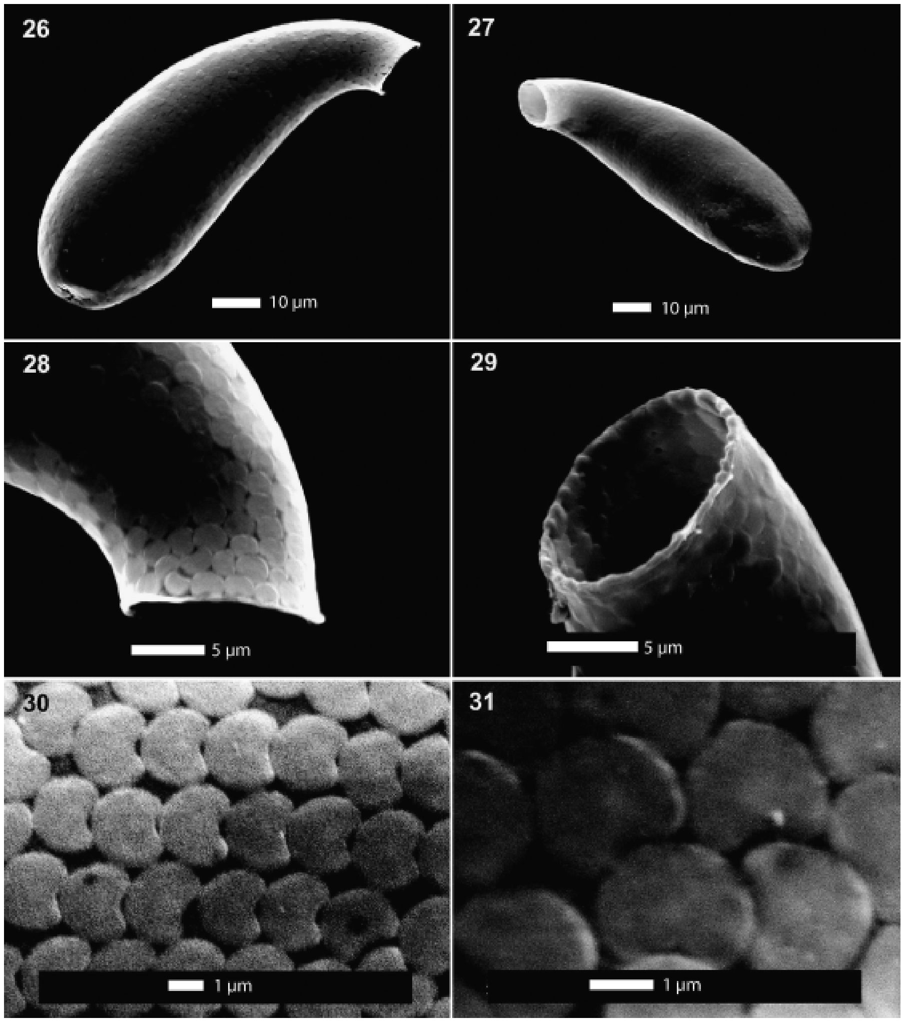

Fig. **26–31.** Scanning electron micrographs of *Cyphoderia compressa* from Black Sea coast (Bulgaria). **26, 27.** Shells; **28, 29.** Detail of the pseudostome; **30, 31.** Detail of the scales arrangement.

(1) test circular in transverse section, composed of non-imbricated disks; (2) test circular in transverse section, composed of imbricated disks; and (3) test triangular in transverse section. Chardez (1991) accepted this classification of *Cyphoderia* shells in his monographic work on the genus.

Our SEM investigations on the shell ultrastructure of eight freshwater and marine (salinity 16–19‰) populations of the genus *Cyphoderia* generally confirmed the above-mentioned peculiarities of shell construction in *Cyphoderia*. However, our study also provides new information on the size, shape, and variability in arrangement of the scales.

The two interstitial psammobiotic testate amoebae (*C. compressa* and *C. littoralis*) are well defined and distinguishable in both biometrical and morphological aspects. Our PCA analysis showed that *C. littoralis* is clearly different from all other species studied in the main shell dimensions and is characterized by having the smallest shell. Additionally this species is further characterised by: (1) the pronounced enlargement in the mid-region of the shell; (2) the strong imbrications of the feeble-convex circular and thin scales; (3) the absence of organic cement on the scales; and (4) the absence of the interstices and perforations between scales. *Cyphoderia compressa* is also a very well-defined and characteristic species. In the original description of the species

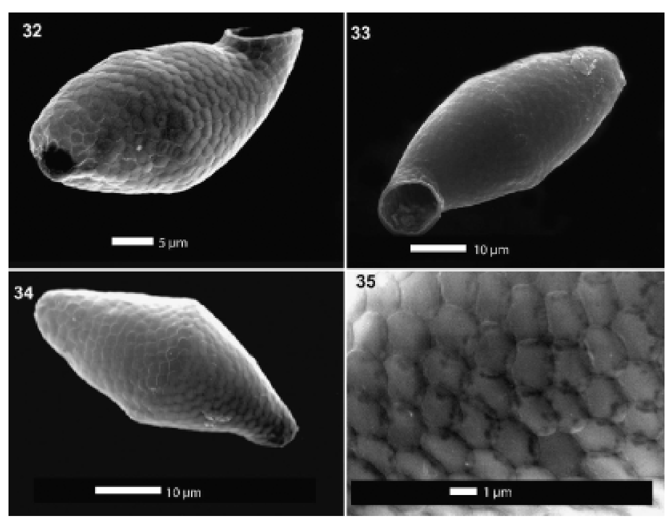

Fig. **32–35.** Scanning electron micrographs of *Cyphoderia littoralis* from Black Sea coast (Bulgaria). **32–34.** Shells; **35.** Detail of the scales arrangement.

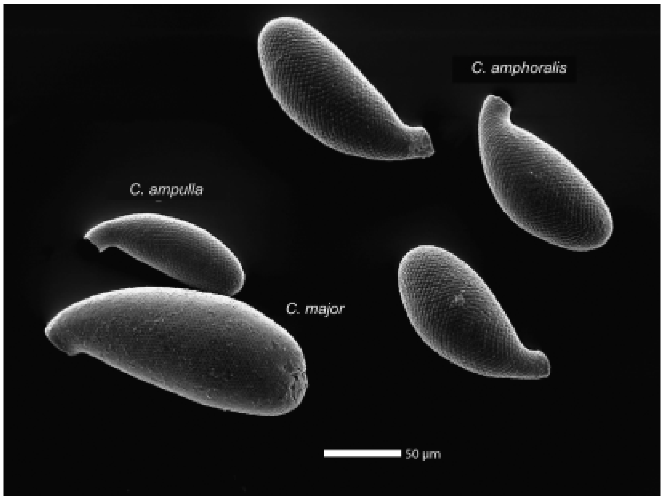

Fig. **36.** Comparative scanning electron micrographs of three different *Cyphoderia* populations; *Cyphoderia amporalis* (Rila), *Cyphoderia ampulla* (Rhodopes) and *Cyphoderia major* (Rila).

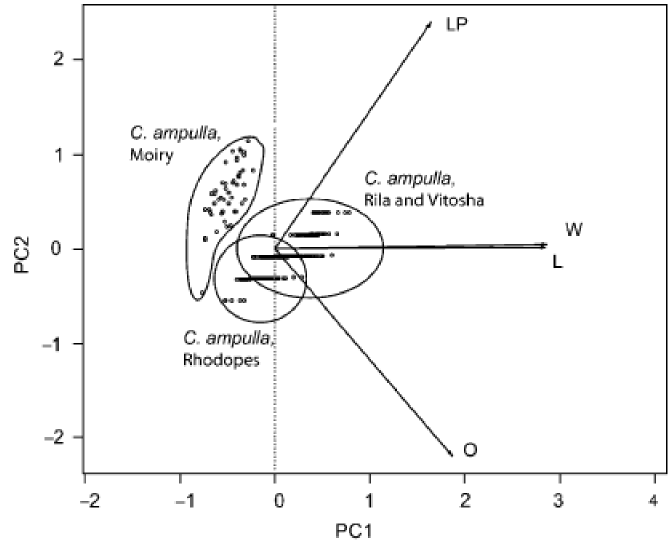

Fig. **38.** Principal component analysis (PCA) based on four morphological traits of four *Cyphoderia ampulla* populations from Moiry, Switzerland and Rhodopes, Rila, and Vitosha, Bulgaria (N = 350). L, length of the shell; W, width; O, degree of overlap of scales; and LP, large axis of the pseudostome.

(Golemansky 1979) and in the publication of Golemansky and Ogden (1980), *C. compressa* is described as similar in outline and in shell structure to the type species of the genus—*C. ampulla*, and differs from it mainly by the strong lateral compression of the shell. Golemansky (1979) noted that ''. . . . le revêtment de la thèque est semblable à ce de *C. ampulla* . . . . . . Dans le cas de *C. compressa* sp. n. le revêtment est formé aussi de petits disques ovales, collés l'un près de l'autre sur la base chitinoide de la thèque (Pl. I 4). Parfois on observe des disques dont la forme n'est pas tout à fait ronde, mais ils ont toujours des bords arrondis (Pl. I 5) . . .''. Almost the same notes were made by Golemansky and Ogden (1980) where the author pointed out that ''. . . . The shell scales of *C. compressa* (Fig. 13) are similar to those of *C. ampulla*, although the number of kidney-shaped scales—about 1.8 µm long and 1.4 µm wide—and the amount of visible organic matrix appears to be greater in the former species . . .'' Our SEM study on the ultrastructure of the shell in *C. compressa* shows that the shell scales in this species are clearly different from all other known *Cyphoderia* species. The shell scales of *C. compressa* are characteristic—kidney-shaped and plane, and clearly differ from the circular, biconvex scales of *C. ampulla* of the Rhodopes, Rila, and Vitosha populations or with the circular or oval plate scales of *C. ampulla* from Moiry.

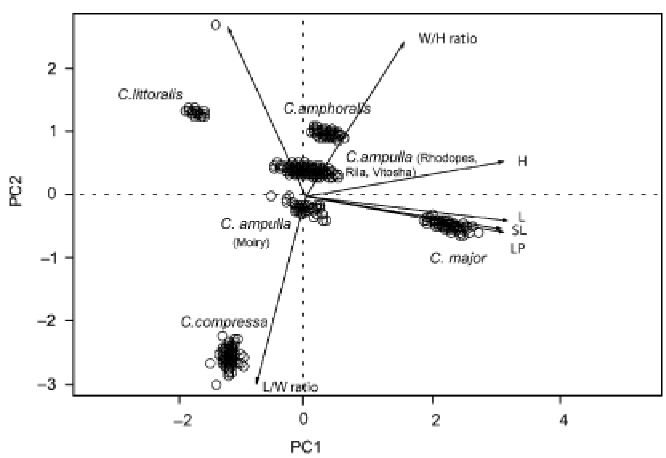

Fig. **37.** Principal component analysis (PCA) based on seven morphological traits of eight *Cyphoderia* populations (N = 750). H, height of the shell; L, length; O, degree of overlap of scales; L/W ratio, shell length/shell width ratio; W/H ratio, shell width/shell height ratio; SL, small axis of the pseudostome; and LP, large axis of the pseudostome.

Besides, we observed little variability in the optical cross sectional views of the test of *C. compressa*. All shells were higher than wide and had an elliptical cross section. In this respect, the shell length/shell width ratio is the most important morphometric character, which clearly distinguishes *C. compressa* from all other *Cyphoderia* species in this study: in *C. compressa* this ratio is about 4 whereas in all other species studied it lies between 1.8 and 2.7 (see Table S1).

Comparative biometrical and ultrastructural study on the three freshwater *Cyphoderia* species (i.e. *C. ampulla*, *C. amphoralis*, and *C. major*) showed that there are well-expressed differences among them. These differences appear clearly when specimens of these species are observed side by side under the microscope (Fig. 8). The most important differences between them can be summarized as follows:

(1) Differences in shell dimensions. Cyphoderia major is the largest of the three species with an average shell length of 192 µm and shell diam. of 78 µm. *Cyphoderia ampulla* shows some size variations among populations, but as a whole, it reaches about two-thirds of the shell dimensions of *C. major* (i.e. shell length: 118–130 µm; shell diam.: 44–50 µm). *Cyphoderia amphoralis* is intermediate between the other two species with an average shell length of 147 µm and an average shell diam. 62 µm.

(2) Differences in size, shape and arrangement of scales. In *C. major* the scales are small (1.6–2.1 µm), circular or oval, plane, non-imbricate and surrounded by a characteristic ring of six perforations around each scale. Specimens of this species correspond to group 1 of the Wailes and Penard's (1911) classification.

Cyphoderia ampulla differs from *C. major* and *C. amphoralis* by having thin, feebly biconvex, circular, and comparatively small (2–3.5 µm) scales, which are arranged side by side or slightly imbricate and are cemented together by abundant organic cement, which frequently extends over the scales. Besides, the scales are almost always equal in size over the whole shell's surface,

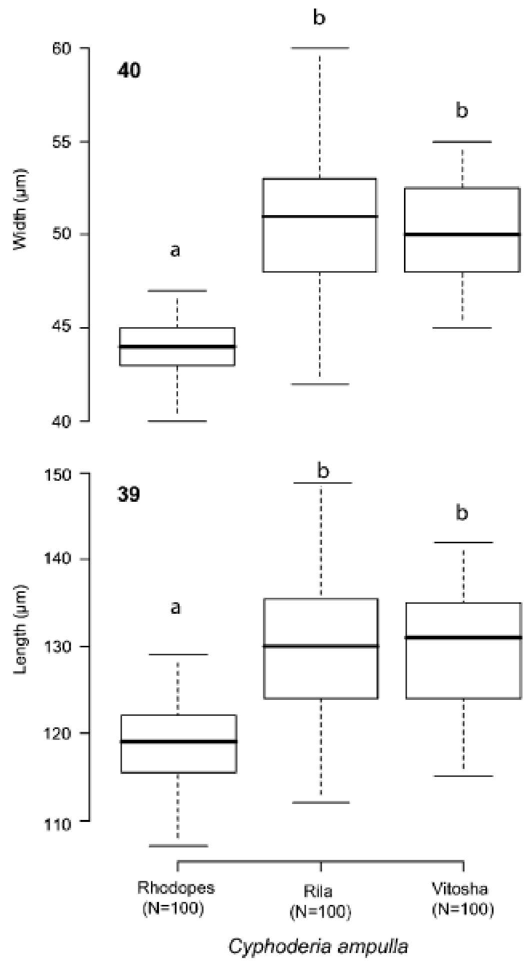

Fig. **39–40.** Box plots representing the length (**39**) and width (**40**) of three *Cyphoderia ampulla* populations from Rhodopes, Rila, and Vitosha, Bulgaria. The boxes represent 50% of the data, limited by the upper and lower quartiles, with the median indicated by a bar within each box. The bars indicate the minimum and maximum values. Different letterings on boxes indicate significant differences between groups as determined by Kruskal–Wallis test using Nemenyi *post hoc* multiple comparisons ($P < 0.05$).

whereas in *C. major* and *C. amphoralis* their size varies between the different parts of the shell. All specimens of the three populations studied correspond well to group 2 of the Wailes and Penard's (1911) classification.

Cyphoderia amphoralis is characterised by the impressively imbricate and comparatively larger (up to 6 µm), circular, biconvex scales. A characteristic for this species is that the shell scales on the mid-region (5–6 µm) are 2–3 times larger than the scales of the apertural and aboral regions (2 = 3 µm).

(3) Shape of pseudostome and number of surrounding apertural scales. Cyphoderia major stands out most clearly in this respect. Its pseudostome is larger (on average 26 µm), always circular, and surrounded by a larger number of apertural scales (35–40). In *C. ampulla* the pseudostome is usually circular, rarely slightly oval, small (on average 16 µm), and is surrounded by 18–22 apertural scales. *Cyphoderia amphoralis* differs from *C. major* and *C. ampulla* by its markedly pronounced oval pseudostome (on average $18 \times 16\,\mu m$) and by a lower number of apertural scales (16–18).

Based on a biometrical analysis and on ultrastructural data for these three freshwater taxa, we conclude that they are morphometrically separable and that the two infrasubspecific taxa *C. trochus* var. *amphoralis* and *C. ampulla* var. *major* should be given species rank as follows: *Cyphoderia amphoralis* n. comb. (Syn.: *C. trochus* var. *amphoralis* Wailes and Penard 1911); and *Cyphoderia major* n. comb. (Syn.: *C. margaritacea* var. *major* Penard 1891).

***Cyphoderia ampulla*—a species complex? – a possible model for biogeography studies?** Our data show that the Swiss and Bulgarian populations of *C. ampulla* are significantly morphologically different. A single population was studied for Switzerland and three different populations for Bulgaria. It would be tempting to interpret this pattern as a possible indication for a biogeographical pattern. The significant morphological differences suggest the possible existence of more than one species. However, a morphological study of four populations does not allow us to assess this with absolute certainty. This raises several questions: (1) Are these different morphologically defined taxa genetically distinct species? (2) If so, how many more species could there be within the ''*Cyphoderia ampulla* species complex''? (3) If these two taxa represent different species, is there more than one species within a given geographical region; in other words is it just by chance that we observed only one morpho-taxa in each of the two countries sampled? These questions illustrate some the current challenges of the study of testate amoeba taxonomy and biogeography that we hope to address in future studies.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Biometric characterisation of the investigated testacean species.