



Description of *Branchiobdella bulgariensis* sp. n. (Branchiobdellida) from Bulgaria

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Abstract: *Branchiobdella bulgariensis* sp. n. is described from the River Topolnitsa near the village of Dushantzi, Bulgaria. The main morphological character distinguishing the new species from *Branchiobdella parasita* (Braun, 1805) is the form of the spermatheca with spermathecal ampulla consisting of almost spherical proximal part and a narrower cylindrical or bulb-like distal part. In contrast, *B. parasita* is characterised by oval spermatheca. Another feature distinguishing these two species from one another is the form of peristomium: in *B. bulgariensis*, the dorsal lip of peristomium has four lobes while, in *B. parasita*, the peristomium is “simple, thin-skinned” (Braun, 1805) and “bears eight micro-tentacles” (Henle, 1835). The only known host of *B. bulgariensis* sp. n. is *Austropotamobius torrentium*. The new species has been found so far at several sites in Bulgaria and unknown locality in Slovakia. An unusual character for the order Branchiobdellida is described, i.e. ontogenetic development of jaws of *B. bulgariensis* resulting in a different shape of these structures in specimens of different age.

Key words: Annelida, Branchiobdellida, *Branchiobdella bulgariensis* sp. n., *Branchiobdella parasita*

Introduction

In the middle of XVIII century, RÖSEL VON ROSENHOFF (1755) reported a small worm living in the gill chamber of crayfishes. Fifty years later, a second species dwelling on crayfishes but on their external surface was described by BRAUN (1805) as *Hirudo parasita* Braun, 1805. ODIER (1823) re-described RÖSEL VON ROSENHOFF'S (1755) species as *Branchiobdella astaci* Odier, 1823 and erected the new genus *Branchiobdella*. Later on, HENLE (1835) published a paper on *B. parasita* explaining that this is BRAUN'S (1805) species *H. parasita*, which should be transferred to ODIER'S (1805) new genus.

The form of chitin jaws was used as a main taxonomic feature for describing European *Branchiobdella* spp. and a main key character for distinguishing them from each other. The early researchers gave very little or no information about the reproductive

system of the species. This is valid also for *B. parasita*, since no details of this system have been presented in either text or drawings in BRAUN'S (1805) paper. The first more detailed information in this respect is given by HENLE (1835). Unfortunately, no type material of *B. parasita* is available (SUBCHEV 2014); therefore, the only sources of information about *B. parasita* are BRAUN'S (1805) and HENLE'S (1835) articles. There are only two subsequent papers containing more details on morphology of *B. parasita* and, in particular, on its reproductive system, i.e., those by KARAMAN (1967) and KOLESNYKOVA & UTEVSKY (2013). However, both papers do not contain comparisons with the original descriptions by BRAUN (1805) and HENLE (1835).

Fifty years ago, the senior author (MAS) presented in his Diploma thesis (published later as KOZAROV et al. 1972) a description of a branchiobdellidan from Bulgaria identified as *B. parasita*. On

Fig. 2 in KOZAROV et al. (1972), the spermatheca of this species was redrawn by publisher and slightly modified – on the original drawing, the spermathecal ampulla consisted of a spherical part continuing distally into a cylindrical part. Another peculiarity found by MAS but not presented in KOZAROV et al. (1972) was that the peristomium of the studied material consisted of two lips divided by a lateral incision, with dorsal lip bearing four small lobes and ventral lip bearing a median emargination. The different shape of the spermatheca in specimens from Bulgaria as compared with HENLE'S (1835) description of this organ requires further studies for clearing up the differences noted in both SUBCHEV'S Diploma thesis and KOZAROV et al. (1962). Unfortunately, the efforts of MAS to collect material from the same locality (village of Lakatnik – river Petreska) for additional examination have failed – at several attempts, no crayfish have been found there. Meanwhile, specimens with similar form of the spermatheca have been collected from other sites in Bulgaria but not in numbers allowing detailed investigations. Only recently, enough numbers of the same branchiobdellidans were collected from another locality in Bulgaria, Topolnitsa River near to the village of Dushantsi. Detailed examinations of branchiobdellidans from the latter locality allowed us to describe the new species presented here.

Materials and Methods

Four crayfish specimens of *Austropotamobius torrentium* (Schrank, 1803) were collected from the river Topolnitsa near the village of Dushantsi (42°41'29.02"N; 24°16'18.50"E; altitude 719 m), South Bulgaria, in September 2017. They were fixed in 70% ethanol. The crayfishes were examined under dissecting microscope in laboratory and all branchiobdellidans found on the surface of the crayfishes as well those in debris of the jar were separated for further examination. An additional sample of branchiobdellidans from the same locality was collected in September 2018. Crayfishes were collected by net and put in a small pail with water containing 30% ethanol for about 10 min. Then each crayfish was shaken vigorously in water for removing branchiobdellidans from its surface before releasing back to the river. The detached branchiobdellidans were collected from the pail bottom, fixed in 70% ethanol and examined in laboratory by dissecting microscope. For further studies, only branchiobdellidans identified provisionally as *B. parasita* were selected.

The external morphology of the selected branchiobdellidans of different age was studied by dis-

secting microscope. In addition, five branchiobdellidans stained in iron acetocarmine (GEORGIEV et al. 1986) were taken through a stepped dehydration series, cleared in dimethyl phthalate and mounted in Canada balsam on microscope slides. As far as preliminary investigations have shown variations in the form of spermatheca and jaws of worms of different age (or size), branchiobdellidans of three groups (small – c. 2 mm, medium-sized – 3–4 mm and large – 6–7 mm long), were dissected and studied. For investigation of the above-mentioned organs and male reproductive system, 12 branchiobdellidans of these three groups were dissected by entomological needles. The dissected organs were placed in glycerine and studied by dissecting microscope. Few dissected jaws from juvenile and adult branchiobdellidans were mounted in Berlese's medium (SWAN 1936). After drying, few weeks after the mounting, the cover slip of the slides were ringed with a nail-polish to avoid further desiccation of the slide. Two dissected spermathecae and two dissected atria with bursa were stained in iron acetocarmine, dehydrated in an ascending alcoholic series, cleared in dimethyl phthalate and mounted in Canada balsam on microscope slides. The observations and the photographs were made using ZeissAxio Imager 2 light microscope equipped with different interference contrast (DIC) and JenopicProgres microscope camera incorporated.

Results

Branchiobdella bulgariensis sp. n.

Type locality: River Topolnitsa, village Dushantsi (42°41'29.02"N; 24°16'18.50"E), Sofia district, Bulgaria.

Type material: Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences: Holotype. IBER Branchio 051, in ethanol. Paratypes: IBER Branchio 052, 30 branchiobdellidans, in ethanol.

Additional material: IBER Branchio 053–057, 5 slides with adults; IBER Branchio 058, IBER Branchio 057, 2 slides with an isolated atrium with bursa; IBER Branchio 058, IBER Branchio 059, 2 slides with an isolated spermatheca; IBER Branchio 060–064, 5 slides with isolated jaws.

Diagnosis: Large branchiobdellidans: fixed specimens up to 7 mm long. Body almost cylindrical; head about 1.2 times wider than segment 1. Peristomium: dorsal lip with four lobes, ventral lip with a median emargination. Jaws triangular, large, their width c. 0.2 of width of head; dorsal jaw with one big median tooth; three pairs of lateral teeth;

ventral jaw slightly smaller than dorsal jaw, with one big median tooth and three pairs of lateral teeth. Spermatheca very big, with a length of spermathecal ampulla reaching up to length of segment diameter. Spermathecal duct short. Spermathecal ampulla consisting of almost spherical proximal part and a narrower (cylindrical to bulb-like) distal part. One pair of testes in 5th segment. Glandular atrium tubular, with a length c. 1.7 times longer than tubular muscular atrium, both folded 3–4 times. Bursa ovoid, with eversible penis covered by small spines.

Description. Fixed smallest branchiobdellidans, most probably juveniles, almost transparent and about 2 mm long or slightly larger. Dorsal part of their large body segments specifically protruded (Fig. 1a). Entrails in live adult branchiobdellidans reddish. Maximum recorded length of fixed mature branchiobdellidans 7 mm (longer specimens can be expected; furthermore, live branchiobdellidans are usually longer than fixed ones). Head c. 1.2 times wider than segment 1. Peristomium divided into two lips by lateral incision (Fig. 1b). Dorsal lip of peristomium bearing four lobes (Fig. 1d). Ventral lip with slight median emargination. The lobes and emargination not always distinct in fixed specimens. Body spindle-like (fusiform) or terete: segments from 1 to or from 1 to 6 gradually slightly increase in width and then again decrease to posterior segment (Fig. 1b, c). Width of sucker almost the same as width of last body segment or slightly bigger.

Measurements of the holotype: body length 6 mm, head length 1.15 mm, head width 0.8 mm, segment 1 width 0.65 mm, segment 5 width 1.1 mm, last segment width 0.9 mm, sucker width 0.9 mm.

In small branchiobdellidans (here and later, we use “young / younger / youngest” and “old / older / oldest” as synonyms of “small / smaller / small-

est” and “large / larger / largest”, respectively), i. e. juveniles and youngest adults, jaws triangular and almost equal in size, with one big median tooth and (usually) three small lateral teeth located almost uniformly along entire lateral sides of jaw (Fig. 2a, b). Small teeth relatively long and sharp. In largest branchiobdellidans, jaws also triangular, with base spanning c. 0.2 times of head width. Ventral jaw slightly bigger than dorsal one. Base of jaws up to 200 µm long. Height of jaws c. 100 µm. Jaws in older branchiobdellidans look differently in comparison with those of young ones – small teeth not located on whole sides but only on fore part of jaw (Fig. 2d, e, f). Jaws thick, with almost triangular lateral view (Fig. 2c). Lateral teeth of largest branchiobdellidans losing their sharpness and resembling pegs.

Female reproductive system presented by spermatheca in 5th body segments and pair of ovaries in 7th body segment. Spermatheca (Fig. 3a, 4) consisting of short spermathecal duct and spermathecal ampulla; very big, with length of spermathecal ampulla reaching length of segment diameter. Duct wide and distinctly shorter than length of ampulla. Spermathecal ampulla consisting of proximal spherical part and narrower distal part. Shape of spermatheca variable, especially with growth of worms; however, the two parts of ampulla always distinct. In youngest mature branchiobdellidans, distal part, which follows spherical part, clearly visible as cylinder, clearly separated from proximal part; equal in length or longer than diameter/length of proximal spherical part (Fig. 4a, b). In oldest branchiobdellidans, distal tubular part shortening, losing its tubular appearance, becoming rather bulb-shaped; border between two parts becoming less distinct (Fig. 3a, 4c).

Male reproductive system consisting of one pair of testes in 5th body segment and tubular glan-

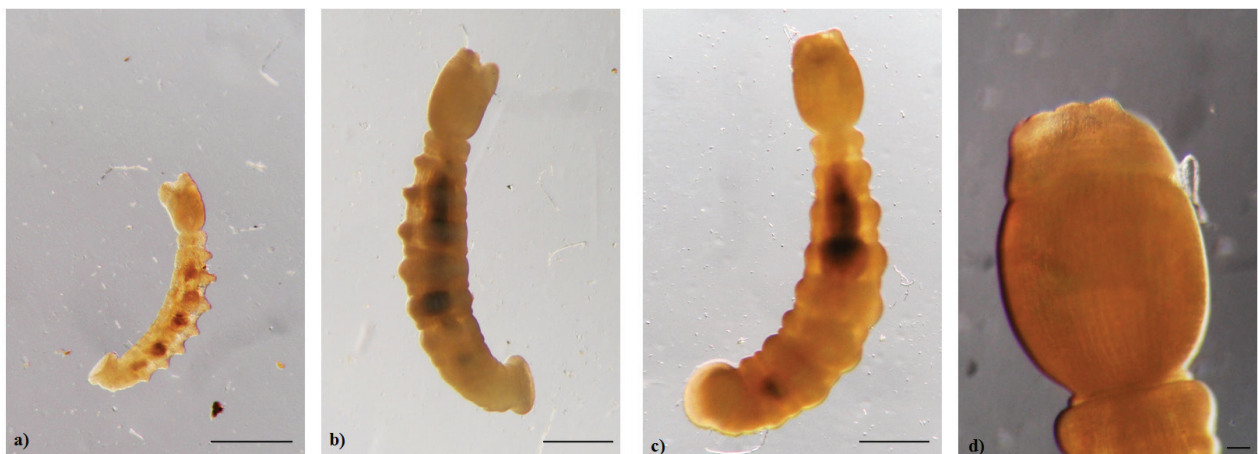


Fig. 1. *Branchiobdella bulgariensis*: a. juvenile; b. adult, lateral view; c. adult (holotype), dorsal view; d. head, dorsal view.

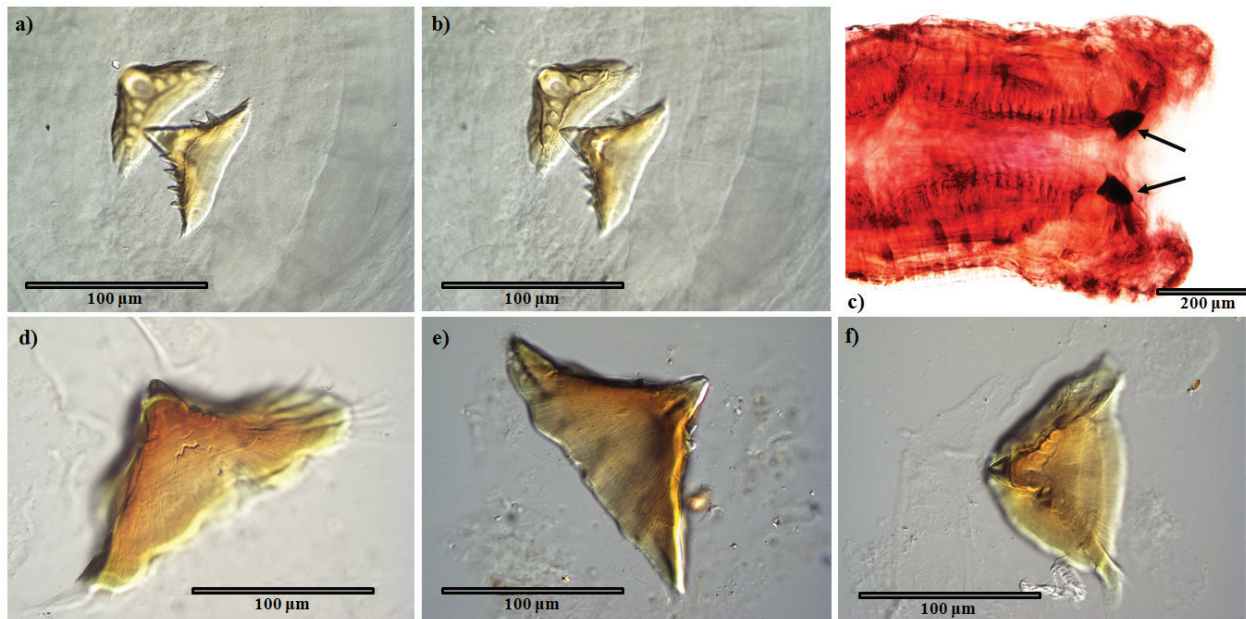


Fig. 2. *Branchiobdella bulgariensis*, jaws: a. juvenile, focused on upper jaw with lateral teeth; b. juvenile, focused on the ventral part of ventral jaw; c. adult, lateral view of jaws (arrows); d. adult, ventral side of dorsal jaw; e. adult, dorsal side of dorsal jaw; f. ventral side of ventral jaw.

dular atrium, tubular muscular atrium and bursa bearing penis inside (Fig. 3b) in 6th body segment. Glandular atrium c. 1.7 times longer than muscular one. Both atria folded 3–4 times within the segment. Muscular atrium ending with ovoid bursa bearing eversible penis covered by small spines.

Variations: The length of the smallest branchiobdellidans is about 2 mm. Small branchiobdellidans, including most probably not only juveniles but also the smallest adults, have specifically arched mid-body big rings (Fig. 1). The fixed mature branchiobdellidans are of different size: in the studied sample, the biggest examined specimens reach 7 mm but, most probably, larger specimens may also exist. The ratio between the width of the big and small body segments varies; usually, the difference in this width is less in smaller worms and better expressed in older specimens.

The number of the lateral teeth in the most cases is three but varies between 2 and 4. With the growth of worms, some of them become blunt and even disappear. Variations of the form of jaws connected with ontogenetic development of worms are presented in the description. Obviously, with the growth of the worms, the jaws grow in their base and thus the little teeth “move” toward.

Variations of the shape of the spermatheca connected with the ontogenetic development are also presented in the description. In some of dissected branchiobdellidans, the bursa was less elongated and had almost spherical form.

Microhabitat: *Branchiobdella bulgariensis* inhabits mainly the ventral side of the abdomen and abdomen appendages, more rarely lateral edges of the carapax. Cocoons are laid on the ventral side of the abdomen and the abdomen appendages.

Distribution: In addition to the type locality, the occurrence of *B. bulgariensis* was proved at four further localities in Bulgaria: river Tsibritsa, Montana Region; river Lalinska, village Lalintsi; river Rakovets, village Samovodene and river Petreska, village Lakatnik (no crayfish found recently) (MAS, unpublished data). A spermatheca very similar by form to that of *B. bulgariensis* was described from a specimen from Slovakia from unknown host and locality (Viera Kilmajerová, MAS, unpublished data).

Host: *A. torrentium*.

Other symbionts on the host: In the type locality and all four other sites in Bulgaria where the occurring of *B. bulgariensis* was proved (see above), numerous *Branchiobdella pentadonta* Whitman, 1882 were also found. Besides *B. bulgariensis* and *B. pentadonta*, a third *Branchiobdella* species, *Branchiobdella astaci* Odier, 1832 was found on the host in the rivers Petreska and Lalinska.

Etymology: For Bulgaria where the species was found and studied. Reference to this country is denoted by the suffix *-ensis*.

Justification: By external view and the shape of jaws, *B. bulgariensis* is similar to *B. parasita* only. The third species with a similar size and tri-

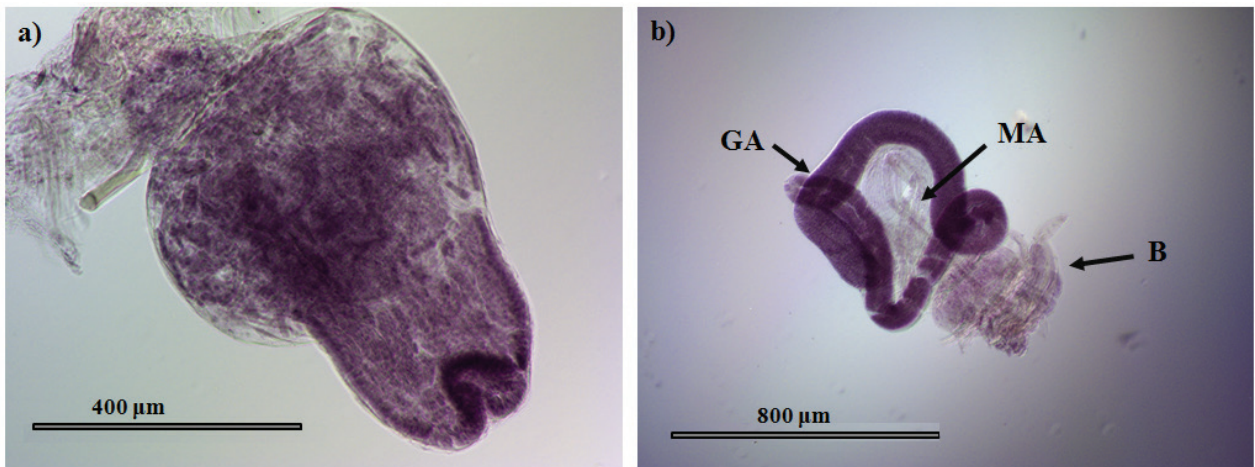


Fig. 3. *Branchiobdella bulgariensis*, reproductive system: a. spermatheca; b. atrium with bursa: B – bursa, MA – muscular atrium, GA – glandular atrium.

angular jaws is *Branchiobdella papillosa* Neesemann & Hutter, 2002; however, the latter species differs clearly from both *B. parasita* and *B. bulgariensis* by the presence of unique body papillae (NESEMANN & HUTTER 2002, SUBCHEV & GELDER 2010). Unfortunately, almost no morphological details for comparison are available in BRAUN (1805) either in the text or in illustrations. The only other source for comparison is the redescription of the species made by HENLE (1835) who gave details of morphology supported by a drawing of whole body.

Both *B. parasita* as described by BRAUN (1805) and HENLE (1835) and *B. bulgariensis* have similar relatively large and almost cylindrical body. The peristomium of *B. bulgariensis* with its four lobes on the dorsal lip differs from the peristomium of *B. parasita* described by the above-mentioned authors. According to BRAUN (1805), the lips of the head are “simple, thin-skinned”. On HENLE’s (1835) drawing (Table XIV), the peristomium of *B. parasita* bears eight micro-tentacles, which have been mentioned also in the text. No visible lobes on the dorsal lip of peristomium are seen on the drawing of the whole body; no such lobes have been mentioned in the text. BRAUN (1805) describes the jaws of *H. parasita* as “dark spots” with a shape of “isosceles triangle”. According to HENLE (1835), the jaws of *B. parasita* are triangular with a big median tooth and three pairs of small lateral teeth, i.e., similar to the jaws of *B. bulgariensis*.

B. bulgariensis and *B. parasita* differ clearly from one another by the form of the spermatheca. An oval ampulla could be seen at the drawing of a whole worm of *B. parasita* (see HENLE 1805). In the text of the latter paper, this organ is described as a “thick-stemmed bladder” followed by a narrower part and a

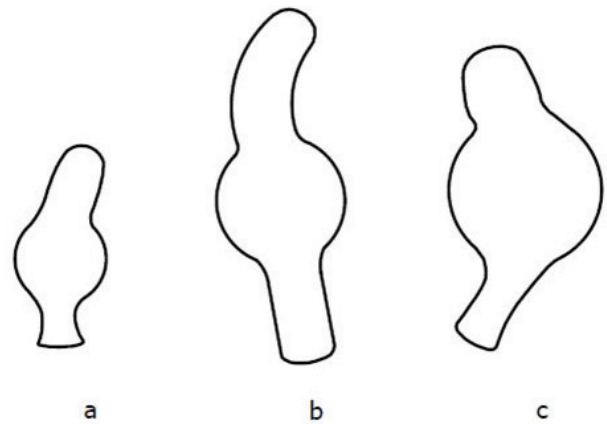


Fig. 4. *Branchiobdella bulgariensis*, schematic view of spermatheca from: a. small specimen; b. medium-sized specimen; c. large specimen.

cylindrical canal. In contrast, in *B. bulgariensis*, the spermathecal ampulla consists of an almost spherical distal part and a narrower proximal part, which is cylindrical in younger specimens and bud-shaped in the oldest ones. The shape of this organ varies with growth only as described above; however, it is never oval. In addition, the spermatheca of *B. bulgariensis* is much larger than the spermatheca presented on the drawing of whole body in HENLE (1835). The length of spermathecal ampulla in *B. bulgariensis* is almost equal to the width of the 5th body ring while the length of HENLE’s (1835) spermathecal ampulla is about ½ of the 5th body segment.

It is difficult to compare the male reproductive system of *B. parasita* as described by HENLE (1805) and that of *B. bulgariensis*. However, it seems that in *B. parasita* (as presented in HENLE’s paper), the glandular atrium is longer than muscular atrium, similarly to that in *B. bulgariensis*.

Discussion

The main key character that undoubtedly distinguishes *B. bulgariensis* from *B. parasita* is the form of the spermatheca. Another difference between the two species is the form of peristomium; however, we cannot exclude that BRAUN (1805) has overlooked the presence of peristomial lobes and HENLE (1835) has presented a miss-interpretation of the shape of peristomium.

As already mentioned, there are only two recent papers presenting original details of the morphology of *B. parasita* and its reproductive system (KARAMAN 1967, KOLESNYKOVA & UTEVSKY 2013). According to KARAMAN (1967), the peristomium of *B. parasita* consists of two parts and thus forms dorsal and ventral parts (no other details are given). The drawing (Fig. 8 in the latter article) is very schematic and, actually, does not coincide with the textual description – no clearly separated dorsal and ventral lips are visible but, instead, the mouth opening is presented by a wave line. In their “diagnosis” of *B. parasita*, KOLESNYKOVA & UTEVSKY (2013) give only: “The dorsal and ventral lips are well marked. The dorsal lip is separated from the head by a deep furrow... The ventral lip is shorter than the dorsal lip”. In the lateral view of adult *B. parasita* in the same paper (Fig. 2a), details of peristomium cannot be seen. As in the case with BRAUN (1805) and HENLE (1835) (see above), we cannot exclude that KARAMAN (1967) and KOLESNYKOVA & UTEVSKY (2013) have overlooked the presence of lobes on the dorsal lip of peristomium in the material studied.

Neither KARAMAN (1967) nor KOLESNYKOVA & UTEVSKY (2013) have studied the changes in the form of the jaws along the growth of the worms. However, different view of jaws of *B. parasita*, corresponding to those described by us in *B. bulgariensis*, were found by VOIGT (1885) who presented two differing jaws of *Branchiobdella varians* var. *parasita* (= *B. parasita*) in his paper. For this variety, he presented jaws with small lateral teeth located uniformly along lateral sides (Table 1, Figs. 6-9) and also a bigger jaw with two small lateral teeth located only on the upper part of its lateral sides (Table 1, Fig. 20), which corresponds to our description of the jaws of youngest and oldest *B. bulgariensis*. At the best of our knowledge, such an ontogenic transformation described in details by us for *B. bulgariensis* has not been described in any other member of order Branchiobdellida.

KARAMAN (1967) stated that spermatheca of *B. parasita* is “a big ovoid capsule with a very short outgoing duct ...”. The organ is also illustrated by a

drawing in his Fig. 12. In addition, the author mentioned a short glandular tentacle at the distal part of the spermatheca, which can be seen also on the drawing. Obviously, the latter structure corresponds to the “ental process” described by HOLT (1960). In the “diagnosis” of *B. parasita* by KOLESNYKOVA & UTEVSKY (2013), it is mentioned: “The spermatheca consists of the oval bulb and very short duct”; this description is confirmed by a photo. Thus, in both cases, the form of spermatheca is closer to the spermatheca described in HENLE (1835) and clearly different from the spermatheca of *B. bulgariensis*.

The description of the male reproductive system of *B. parasita* is quite superficial in KARAMAN (1967) and is lacking in the text by KOLESNYKOVA & UTEVSKY (2013); in the latter case, it is presented only as a picture. However, in both cases the glandular atrium seems to be longer than the muscular atrium, e.g., similar to that in *B. bulgariensis*.

It is clear that the description of *B. parasita* presented in KARAMAN (1967) and KOLESNYKOVA & UTEVSKY (2013) is much closer to the description of this species by HENLE (1853) than to that of *B. bulgariensis*, especially in regards to the shape of spermatheca, being oval in all three cases. The only difference in regards to the latter organ is the presence of a narrowing portion of the spermathecal ampulla in its proximal part described by HENLE (1853) and the absence of such narrow part in the corresponding descriptions by both KARAMAN (1967) and KOLESNYKOVA & UTEVSKY (2013). It is not clear also if the presence of ental process on the spermatheca of *B. parasita* redescribed by KARAMAN (1972) is enough for stating about some significant differences between his material and the material studied by KOLESNYKOVA & UTEVSKY (2013). Furthermore, according S. GELDER (personal communication), this structure may be absent due to preservation by some chemicals.

HOFFMAN (1966) published a short description of *B. parasita* (misspelled by him as *B. parasitica*) collected on crayfishes from the Lake Szhvarzegrønn, Luxembourg, and results of his laboratory observations on the collected branchiobdellidans. The peristomium of *B. parasita* presented as a drawing in the paper looks exactly like that of *B. bulgariensis*; it has four lobes on the dorsal lip and median incision on the ventral lip. However, there are two unusual peculiarities on the body drawing – there are two small rings before the first large one and the width of the sucker is almost twice wider than the last body segment. In the text, only the triangular jaws and penis with many spikes on its apex located in a “big oval sac” were mentioned.

It worth mentioning also that the second specimen presented as a drawing of *B. parasita* on fig. 9 in NESEMANN & NEUBERT (1999) also resembles *B. bulgariensis* by barely distinguishable lobes on the dorsal lip of peristomium. The peristomium of this specimen (lower Austria, outflow of the Altenburger Stifisteich) differs well from the peristomium of the specimen from Bavaria, Wielenbach, presented on the same figure. Thus, in the case that four-lobed dorsal lip of peristomium, there is a specific character only for *B. bulgariensis* but not for *B. parasita*. Therefore, a wider distribution of *B. bulgariensis* could be expected.

The case with the morphologically close *B. parasita* and *B. bulgariensis*, distinguishable mainly by the form of spermatheca, is not unique for European *Branchiobdella* spp. KARAMAN (1970) differentiated so called “*pentadonta* group” comprising three species with the same form of jaws: *Branchiobdella pentadonta* Whitman, 1882, *B. italica* Canegallo, 1923 and *B. balcanica* Moszyński, 1937. All these species have jaws with almost rectangular base plate and normally five jaws with the median and lateral ones being larger; in *B. pentadonta*, the number of the teeth could vary up to seven (HALGOŚ 1972, KOZAROV et al. 1972) and such variations are known also for *B. italica* and *B. balcanica* (see SUBCHEV 2014). Actually, *B. balcanica* is well distinguishable from *B. pentadonta* and *B. italica* by its fourth body segment, clearly larger than the preceding third one. As to *B. pentadonta* and *B. italica*, according to GELDER et al. (1964) “The only character to separate the two species [*B. pentadonta* and *B. italica*] reliably is the ratio of the spermathecal duct to bulb length”. The case with *B. parasita* and *B. bulgariensis* is similar, i.e. these two species possess very similar triangular jaws with a big median tooth and two - four lateral teeth but the form of the spermatheca in the two species with clearly different form is a stable and reliable character to distinguish undoubtedly them each from other. Neither KARAMAN (1967) nor KOLESNYKOVA & UTEVSKY (2013) mentioned about any variability of the oval form of spermathecal ampulla. Similarly, no oval form of the spermatheca in *B. bulgariensis* was found in the numerous studied specimens from type locality and the other localities in Bulgaria where this species was found.

It is interesting to note that the molecular study by SARIĆ et al. (2018) on European *Branchiobdella* spp. predicted a possible occurrence of cryptic “*parasita*” species. The authors found three distinct haplogroups for *B. parasita* from different localities and hosts and concluded that the high value p-distances

between two of them, G6 and G8, ranging from 8.6 to 12.3%, suggests that the latter may represent cryptic species. For comparison, the p-distances given in the same paper for *B. pentadonta* and *B. italica* is 10.9%.

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