

New species of aquatic oligochaetes (Annelida: Clitellata) from tufa barriers in Croatia

PILAR RODRIGUEZ^{1,*}, NATALIJA VUČKOVIĆ² & MLADEN KEROVEC²

¹Department of Zoology and Animal Cell Biology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Box 644, Bilbao, 48080 Spain.

²Department of Zoology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia

*Corresponding author. E-mail: pilar.rodriguez@ehu.es

Abstract

Two new aquatic oligochaete species are described from tufa barriers in Croatia. The reproductive system in the phallodriline *Aberrantidrilus mihaljevici* Vučković *et al.* **n. sp.** conforms to the general pattern in the genus but it is distinguished by a combination of characters, including the shape and length of penial chaetae (3 times the mean length of somatic chaetae), atrium shape and spermathecal pore position (anterior in the postatrial segment). The lumbriculid *Stylodrilus tofaceus* Rodriguez *et al.* **n. sp.** is mainly distinguished from congeners by the spindle-shaped spermathecal ducts with high lining epithelium, and position of the spermathecal pores. These two new taxa reveal that aquatic oligochaete communities in tufa barriers of karstic regions may be of particular interest with respect to diversity of endemic taxa, and also for study of colonization routes of this interesting habitat, with possible relations with groundwaters. The finding of one specimen attributable to the genus *Eclipidrilus* may open a interesting research on the biogeography of this Nearctic genus.

Key words: *Aberrantidrilus*, *Stylodrilus*, tufa, Dinaric region

Introduction

The Dinaric karstic region of the western Balkan Peninsula is known for high diversity of different aquatic karstic habitats (Sket 2005). Tufa barriers are some of the unique hydrogeological structures associated with karst waters, usually forming barrage lakes on a longitudinal profile of karstic rivers. Tufa is a porous calcium carbonate deposit that develops mainly in near ambient temperatures or cool freshwaters, where heavy calcite precipitation occurs on organic substrates, associated with inorganic processes, as well as with microbial communities dominated by diatoms, bacteria and cyanobacteria (Riding 1991; Pedley 2000). This specific calcite precipitation is particularly related to biofilm on mosses that provide a substrate for calcite nucleation (Golubić *et al.* 2008). The growth of microorganisms, combined with carbonate deposition, produces a porous, rapidly hardened substrate (Golubić *et al.* 2008). Irregular calcite deposition and distribution patterns of particulate organic matter along the flow results in varying microhabitats in tufa barriers (Miliša *et al.* 2006), with high potential biodiversity, e.g. in insect communities as reported by Ivković *et al.* (2012).

Before the discovery of *Branchiura sowerbyi* Beddard, 1892 in a pond of the Botanical Garden in Zagreb in 1955, there was no research conducted on aquatic oligochaetes in Croatia (Devidé 1956). After this finding, extensive research started on karst rivers, lakes (Matoničkin & Pavletić 1961, 1962, 1964) and other freshwater habitats in Croatia (Hrabě 1973; Kerovec 1980, 1981a, b, 1985a, b, 1990, 2002; Mihaljević *et al.* 2001, 2007; Kerovec & Kerovec 2014; Martínez-Ansemil *et al.* 2016). Although Croatia is a relatively small country, high oligochaete species richness is a result of Croatia's geographical position, which includes two sea basins and two ecoregions (Illies 1978). A checklist of Croatian freshwater oligochaetes, based on literature records and unpublished records from research conducted over the past four decades, was compiled and published by Kerovec *et al.* (2016). This checklist included 71 recorded species and 35 in the Krka River basin, of which 19 species were found on Roški slap tufa barriers. It is interesting, however, to point out that the present contribution is the first to describe new aquatic oligochaete taxa from the tufa barriers.

The aim of this study is to contribute to the knowledge of the fauna inhabiting tufa habitats with the description of two new oligochaetes species and a discussion of the possible relationships with groundwater fauna.

In tufa barriers of Croatia, several aquatic oligochaetes species are known to date, namely, 13 species of Naidiinae, six of Tubificinae, and one species each of Pristininae, Lumbriculidae, Propappidae, and Lumbricidae (Kerovec *et al.* 2016). In the following, we provide the description of a new species of *Aberrantidrilus*, a genus that belongs to Phallodrilinae, a taxon not previously reported in this habitat, and a new species of *Stylodrilus*, family Lumbriculidae. From the same habitat, we describe one further specimen of Lumbriculidae of uncertain species identity and assigned to *Eclipidrilus*. In an appendix, we give an overview of the freshwater species of Phallodrilinae, a subfamily that is predominantly marine.

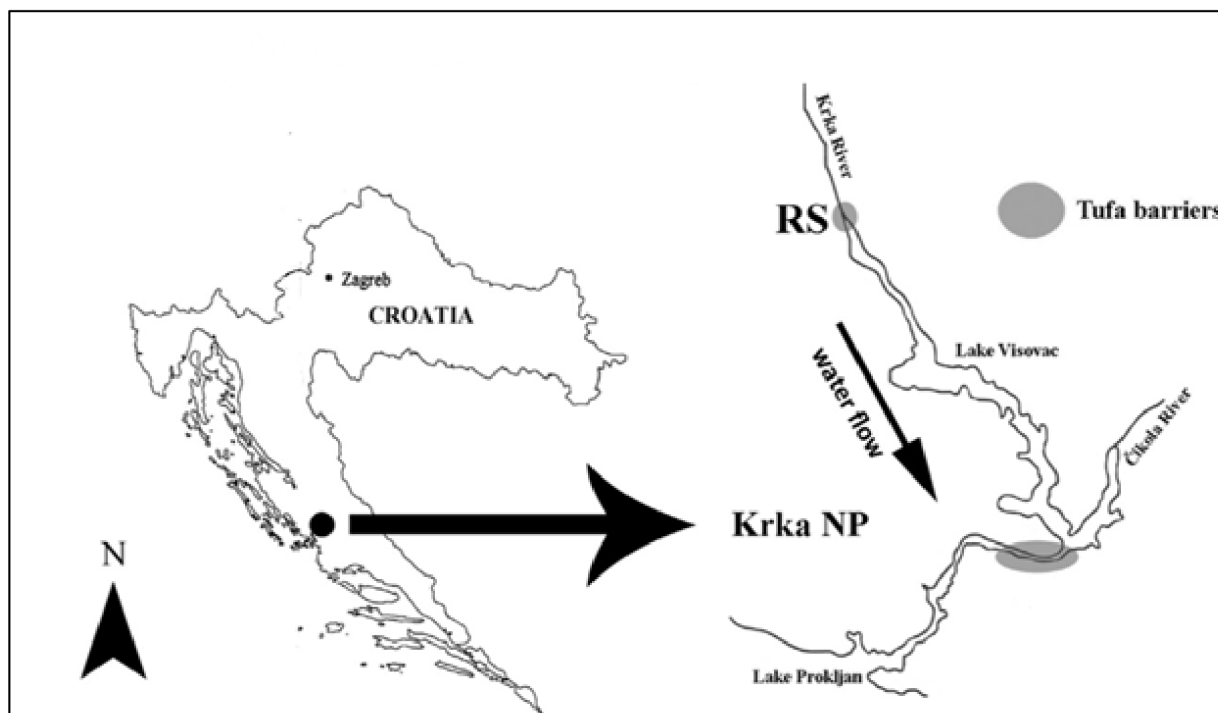


FIGURE 1. Study area. Abbreviations: RS, Roški slap; Krka NP, Krka National Park.

Material and methods

From October 2013 to October 2014, a sampling program was conducted in Roški slap, situated in the Krka National Park (Fig. 1), located northeast of the town of Knin. The Krka River is 72 km long, with a few barrage lakes along its course, of which Lake Visovac, located downstream the study area, is the largest and has previously been investigated for aquatic oligochaetes (Mihaljević *et al.* 2001). There are seven tufa barriers with partially moss-covered microhabitats along its length, including Roški slap. Downstream from the tufa barrier, water is fast-flowing and supersaturated with calcium salts, DOM concentration is lower than 10 mg C L⁻¹ and pH is higher than 8.0 (Srdoč *et al.* 1985; Srdoč 1990; Stilić & Božićević 1998).

Samples for a global study on the macroinvertebrate community were collected with a 100 cm² core sampler, to 5 cm sediment depth, minimally disturbing the sites. The tufa sampled is submerged in water all along the year, at less than 30 cm depth from the water surface. At each location, three replicate subsamples were collected and sieved through a 200-µm mesh size. Collected samples were fixed in 96% ethanol, stored in bottles, and transported to the laboratory. Oligochaetes were separated from the samples under a stereomicroscope and conserved in 80% ethanol for further identification and taxonomic studies. Specimens used for the morphological descriptions were stained in Ehrlich's hematoxylin, followed by differentiation in 1% acid (HCl)-ethyl alcohol 70%, dehydrated in an alcohol series, cleared in methyl salicylate, and mounted in Canada balsam.

A collection of specimens of *Bythonomus sulci* Hrabě, 1932 was loaned by the Museum of Natural History of Prague and was examined for comparative purposes with the new *Stylodrilus* species described here. For the same

purpose, material (whole-mounts and dissected specimens) of *Stylodrilus lemni* (Grube, 1879), from the personal collection of the first author, was reinvestigated.

The type material is deposited at the Museo Nacional de Ciencias Naturales (MNCN), CSIC, Madrid, Spain. Non-type material is in the personal collection of the first author.

Abbreviations. a—atrium, aa—atrial ampulla, ad—atrial duct, fp—female pore, m—musculature, mp—male pore, mvl—mid ventral line, o—ovary, p—penis, pc—penial chaeta, pph—male porophore, pr—prostate, sa—spermathecal ampulla, sd—spermathecal duct, sf—sperm funnel, sp—spermathecal pore, t—testis, vch—ventral chaetae, vd—vas deferens.

Results

Family Naididae Ehrenberg, 1828

Subfamily Phallodrilinae Brinkhurst, 1971

Genus *Aberrantidrilus* Martin, 2015

Aberrantidrilus mihaljevici Vučković, Rodríguez & Kerovec n. sp.

(Figures 2, 3)

Holotype. MNCN16.03/3103, one whole-mounted individual, stained with hematoxylin and mounted in Canada balsam.

Paratype. MNCN16.03/3104, one whole-mounted individual, stained in hematoxylin and mounted in Canada balsam, from type locality.

Type locality. In the tufa barrier of Roški slap, Croatia. April 28, 2014. Coordinates: 43°54'12.1"N, 15°58'31.3"E (43.90336 N, 15.975348 E). Collector: Zlatko Mihaljević.

Etymology. The species is named to honor its collector, Dr. Zlatko Mihaljević, professor of Zoology at the University of Zagreb, for his interest and contribution to the study of the biota in tufa barriers of Croatia.

Description. Specimens posteriorly incomplete, number of segments 14 (paratype) and 33 (holotype). Length of the longest worm 3 mm; maximum diameter 234 and 248 µm in segments IX and XI, respectively. Prostomium 71 µm long and 80 µm wide at its base (Fig. 2A). Body wall consisting of a thin cuticle, an epidermal layer (4–7 µm thick) and musculature (5–6 µm thick). Clitellum not raised, formed by epithelial glands in transverse lines, from segment X behind the chaetal bundles to the anterior third of XII. One pair of simple spermathecal pores in the most anterior part of segment XII, opening in line with ventral chaetae. One pair of male pores in segment XI on porophores (36 µm high, 61 µm wide), in line with ventral chaetae. Dorsal and ventral chaetae bifid in anteclitellar segments, simple-pointed from XIV; in XII and XIII chaetae simple-pointed in dorsal bundles and bifid in ventral bundles (Figs. 2B,C). Anterior segments with (3)4–7 chaetae per bundle and postclitellar segments with 5–7; chaetae usually more numerous in II and in the posterior segments (Table 1). Length of chaetae gradually increasing from II to VI (34–44 µm), with distal tooth about the same length as proximal, and nodulus about median. Chaetal bundles situated in the posterior third of the segment. Penial chaetae occurring singly in segment XI, sigmoid, the tip grooved like the tip of a hypodermic needle (Fig. 2H), or frayed, spatulate tip (only seen when mounted in Canada balsam) (Fig. 2F, G), and oriented antieriad; length 117–124 µm, 2.7–3.2 times the length of somatic chaetae, and thickness 4 µm.

No coelomocytes observed within the coelomic cavity. Pharyngeal glands in segment III dorsal to the pharynx, and from IV–VI both dorsal and ventral to the oesophagus. Chloragogen cells from segment VI, backwards. The beginning of the intestine well-defined by a dilatation of the gut in VIII. First nephridia in 6/7 (Fig. 2J), and additional nephridia observed in some posterior segments.

One pair testes in segment X and one pair ovaries in segment XI (Fig. 3). Anterior sperm sacs extending to segment IX and posteriorly to XII, ovisac to XIII containing yolk (one oocyte also visible in the holotype). Male ducts paired. Vas deferens (diameter 6–8 µm), ciliated, shorter than the atrium (Fig. 2D), junction with atrium not clearly determined, although it appears to be at the ental end of the atrium. One large prostate gland attached to the ental end

of the atrial ampulla; a second prostate gland is not seen. Atrial ampulla sac-like (154–162 μm long, diameter 83–92 μm) and bent; atrial duct 43–46 μm long, diameter 36 μm . Atrial musculature very thin (2–3 μm), atrial epithelium 5–17 μm , thicker in the ectal section. Each atrium opens in a prominent round porophore (36 μm long, diameter 61 μm), where the penial chaeta is located (Fig. 2E, G, H). Penis can be protruded from the porophore, length up to about 22 μm (Fig. 2E).

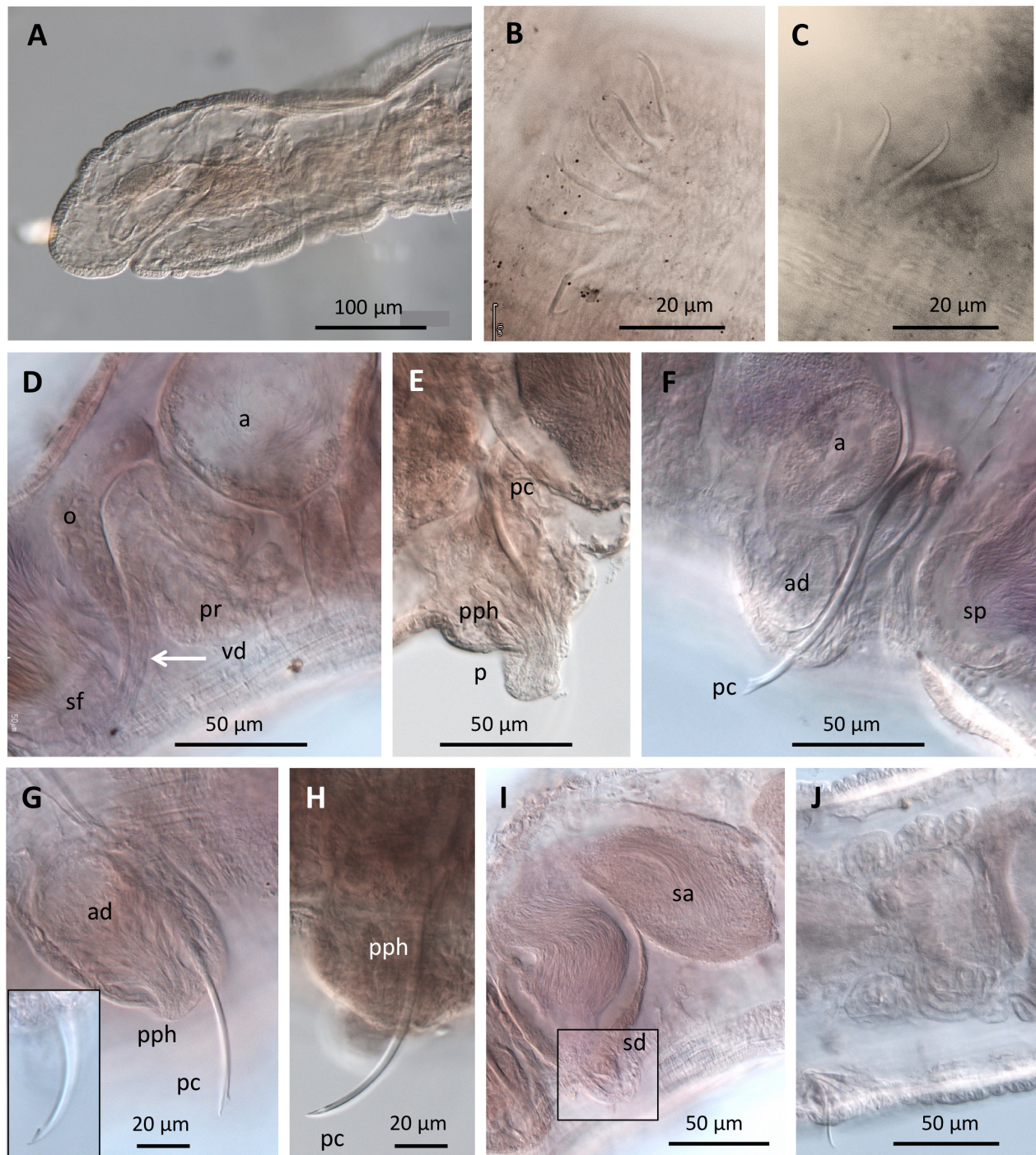


FIGURE 2. *Aberrantidrilus mihaljevici* n. sp. **A.** Anterior part of the body. **B.** Bifid chaetae in segment V. **C.** Simple-pointed chaetae in postclitellar segment. **D.** Male duct and ovary in segment XI. **E.** Porophore and penis extruded in segment XI. **F.** Penial chaeta and distal part of atrium in segment XI, spermatheca in front of segment XII. **G, H.** Penial chaeta showing frayed, spatulate (G) and grooved distal tip (H). **I.** Spermatheca in XII, within the square the short spermathecal duct as photographed on a slightly different focus plane of the same spermatheca. **J.** Nephridial ducts in segment VII.

One pair spermathecae with a very short, conical duct (27 μm long, 22 μm wide) and an elongate, globular ampulla (153–166 μm long, 106 μm maximum diameter), which contains a mass of sperm without forming discrete sperm bundles (Fig. 2I).

Remarks. The new species is clearly ascribed to the genus *Aberrantidrilus* Martin, 2015 by the chaetal characteristics (simple pointed and bifid), position of the spermathecae in the postatrial segment, porophore and penis associated with a single, penial chaeta with the tip oriented forward. Our study material is limited, and the fact that an ectal prostate gland has not been seen in the new species is probably due to its small size, as for example in *A. cuspis* (Erséus & Dumnicka, 1988).

Prior to Martin *et al.* (2015), this new species would have been placed in *Abyssidrilus*, a genus erected by Erséus (1992) to include species of the subfamily Phallodrilinae with slender, simple-pointed and/or bifid chaetae, vas deferens entering the apical end of the atrium, and prostate glands attached separately at the most ental and most ectal ends of the atrium. However, Martin *et al.* (2015) erected the new genus *Aberrantidrilus* to accommodate all the freshwater subterranean species formerly classified as *Abyssidrilus* Erséus. In their review on the worldwide diversity of freshwater oligochaetes, Martin *et al.* (2008) reported 9 phallodrilina genera, which included both marine and freshwater species. In this context, the validity of the genus *Aberrantidrilus* is stressed by the fact that the freshwater species formerly included in *Abyssidrilus* are clearly separated from the marine species (which remain in *Abyssidrilus*) by the position of the spermathecal pores in the postatrial segment (XII), and by the presence of a single penial chaeta per bundle with the tip oriented forwards (Martin *et al.* 2015). The marine *Abyssidrilus* species have typically 2 penial chaetae [except in *A. stilus* (Erséus, 1986) with only one penial chaeta], which are oriented backwards, as indicated by Martin *et al.* (2015). Moreover, additional modified chaetae in spermathecal and pre-spermathecal segments (IX and X segments) are present in three out of nine *Abyssidrilus* species, but are absent in *Aberrantidrilus*. Finally, another interesting diagnostic and probably apomorphic character in *Aberrantidrilus* is the presence of ventral protuberances or porophores at the male pores, although, once more, the abyssal marine species *Abyssidrilus stilus* shares this character with the *Aberrantidrilus* species.

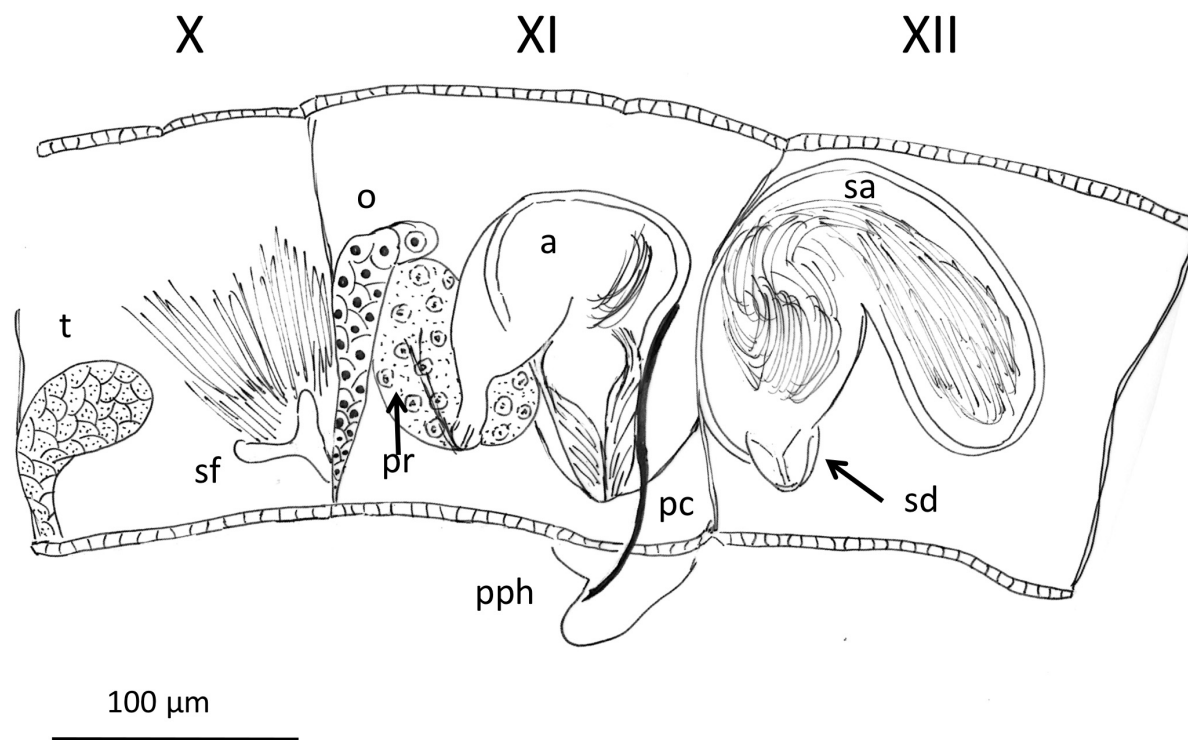


FIGURE 3. *Aberrantidrilus mihaljevici* n. sp. Schema of the reproductive segments.

Compared with the previously described *Aberrantidrilus* species from subterranean freshwater habitats (Table 2), the new species has the longest penial chaetae (3 times the mean length of somatic chaetae). Furthermore, the number of chaetae in posterior segments is higher in *A. mihaljevici* n. sp. than in congeners (5–7 vs up to 4). Total

length of the atrium appears to be within the range of other species described so far, but here the atrium has a greater diameter due to its sac-like shape, so its volume is larger than in congeners.

TABLE 1. Number (N) of chaetae per bundle and length (L) in 2 specimens of *A. mihaljevici* n. sp. (–, not possible to measure due to orientation of chaetae).

Segment	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	posterior
Dorsal (N)	6	4–5	5	5	5	3–5	4	4	4	1, 4	1, 3	6–7
(L)	34	38	38	–	43	42	41	–	–	–	40	43
Ventral (N)	5–7	6	6	4–6	4–6	4–5	3–4	4	4	1	3–4	5–6
(L)	35	36	44	41	42	43	–	–	–	117–124	–	41

The new species is similar to *A. cuspis* in the shape of somatic chaetae, bifid anteriorly and simple-pointed posteriorly, but the spermathecal pores are located near septum 11/12 and in the longitudinal line of the ventral chaetae, whereas in *A. cuspis* they are located near the middle of the segment and laterally to the line of the ventral chaetae. Further comparison of *A. mihaljevici* n. sp. with *A. cuspis* is made difficult by apparent variability of characters and uncertain species boundaries of the latter. For example, the penial chaetae of *A. cuspis* are originally described as simple-pointed, whereas in Sambugar *et al.* (1999) they are bifid. These authors also report on a number of specimens with uncertain identity, identified as *A. cf. cuspis*, but with penial chaetae small or “may be absent”. The problematic variability of *A. cuspis*, which also probably includes material from Slovenia identified as “*Abyssidrilus* sp. 1” by Giani *et al.* (2011), is presented and discussed in Martin *et al.* (2015), who hypothesize that *A. cuspis* may represent a complex of cryptic or “near-cryptic” species.

Family Lumbriculidae Vejdovský, 1884

Genus *Stylodrilus* Claparède, 1861

Stylodrilus tofaceus Rodriguez, Vučković & Kerovec n. sp.

(Figures 4, 5)

Holotype. MNCN 16.03/3105, one sagittally dissected individual, posteriorly incomplete, fully mature, stained in hematoxylin and mounted in Canada balsam, 6 November 2013.

Paratype. MNCN 16.03/3106, one sagittally dissected, fully mature individual, and MNCN 16.03/3107 one whole-mounted, with developed reproductive organs but unmated individual; both paratypes are complete, stained in hematoxylin and mounted in Canada balsam. From type locality, 6 November 2013.

Type locality. Roški slap, Croatia, Coordinates: 43°54'12.1"N, 15°58'31.3"E (43.90336 N, 15.975348 E). Collector: Zlatko Mihaljević.

Etymology: species named from Latin “tôfus”, a term applied to several soft rocks including the calcareous tufa.

Further material investigated. 3 dissected individuals and 4 whole mounts, all fully mature, except for one whole-mount with developed reproductive organs but unmated, stained and mounted in Canada balsam, 6 November, 2013. 29 worms sampled in 6 November, 2013 and 15 worms sampled in 7 February, 2014, kept in alcohol 70%; all from the type locality but poorly fixed and unsuitable for histological studies (in P. Rodriguez collection).

Description (based on fully mature individuals, i.e. with sperm in spermatheca and/or atrium). Segment number 53–69 (on 4 complete individuals, including both paratypes). Diameter in segment X, 0.60–0.70 mm. Prostomium rounded, 138–217 µm long. Secondary annulations not visible in preclitellar segments, slightly marked from XI backwards (although perhaps due to poor fixation). Chaetae simple-pointed, two per bundle (Fig. 4A–C). Anterior ventral chaetae 80–125 µm long, shortest in II, longer in posterior bundles (117–132 µm); chaetal width 4–6 µm (thinner in II, c. 3–4 µm); nodulus at about 0.4–0.5 from distal end; in the same segment, dorsal chaetae shorter than ventrals. In the rearmost section of the body (from about segment XXXV), chaetae thicker (5–7 µm) and distally more curved (Fig. 4C), with nodulus generally at one third from the tip of the chaeta. In the partially matured worms the same pattern is observed.

TABLE 2. Comparison of morphological characters of *Aberrantidrilus* species. Abbreviations: ant, anterior(ly); post, posterior(ly); (–) character not reported in the descriptions. In the column of *A. cuspis* sensu Sambugar *et al.* (1999), data of “*A. cf cuspis*” are excluded; see text for further explanations.

	<i>A. cuspis</i> (Erséus & Dumnicka, 1988)	<i>A. cuspis</i> (Erséus & Dumnicka, 1988)	<i>A. stephaniae</i> Martin, 2015	<i>A. subterraneus</i> (Rodríguez & Giani, 1989)	<i>A. mihaljevici</i> n. sp.
Data from	Erséus & Dumnicka (1988)	Sambugar <i>et al.</i> (1999)	Martin <i>et al.</i> (2015)	Rodríguez & Giani (1989)	this contribution
Max. body width [µm]	190–280	128	139–164	127	234–248
Number of penial chaeta, (length, [µm])	one (100–120)	one (50–60)	one (50–64)	one –	one (117–124) /
Penial chaetae distal end	simple-pointed	bifid	bifid	simple-pointed	spatulate or grooved simple-pointed
Number of somatic chaetae in anterior / posterior segments	2–5 (7) / 2–4	3–6	2–5 / 1–3	2–5 / 3	1–7 / 5–7
Shape of somatic chaeta	bifids ventrally, similar tooth length; upper tooth reduced or absent in post. dorsals	bifids, similar tooth length ant.; upper tooth reduced or absent post.	bifids, similar tooth length	bifids, upper tooth reduced	bifids, similar tooth length ant.; simple pointed post.
Length ratio of penial to somatic chaetae	mean: 2.3	mean: 1.2–1.8	1.4	–	Mean: 3.0
Atrium: length [µm] / max. diameter [µm]	275–350 / 28–40	–	207–287 / 39–47	100 / 30	197–208 / 92
Spermathecal pore position in segment XII	near middle (lateral to the line of ventral chaetae)	near middle (lateral to the line of ventral chaetae)	near middle (lateral to the line of ventral chaetae)	near septum 11/12 (–)	near septum 11/12 (in the line of ventral chaetae)
Habitat and location	subterranean waters in wells in Central Italy	subterranean waters in North Italy, France	hyporheos in West France	Cave in North Spain	Tufa in Croatia

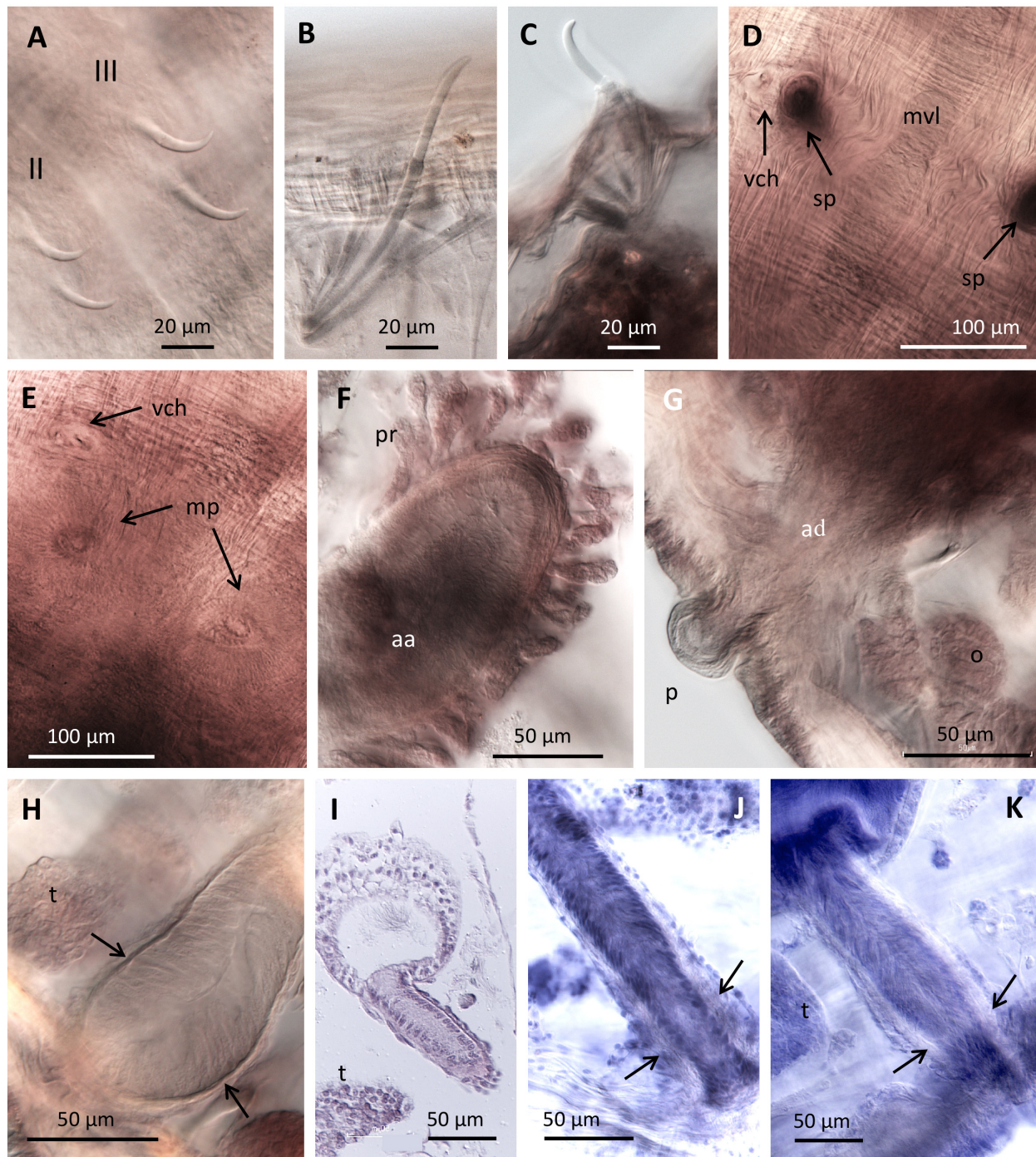


FIGURE 4. *Stylo-drilus tofaceus* n. sp. (A–H), *S. sulci* (Hrabě, 1932) (I) and *S. lemani* (Grube, 1879) (J, K). *S. tofaceus* n. sp.: A. Ectal ends of ventral chaetae in segments II and III. B. Dorsal chaeta in XI. C. Posterior chaeta. D. Spermathecal pores in IX in the transversal plane of ventral chaetae in IX. E. Male pores in X. F. Ental part of atrial ampulla, covered by small clusters of prostatic cells. G. Atrial duct and penis. H. Spermathecal duct. I. Spermathecal duct in *S. sulci* (Hrabě), from Hrabě's original series, section on slide, Museum of Natural History of Prague. J, K. Spermathecal duct in *S. lemani* (Grube), specimens from northern Spain, personal collection of P. Rodríguez. Arrows in H, J and K point to the muscular layer of the spermathecal ducts for comparative purposes.

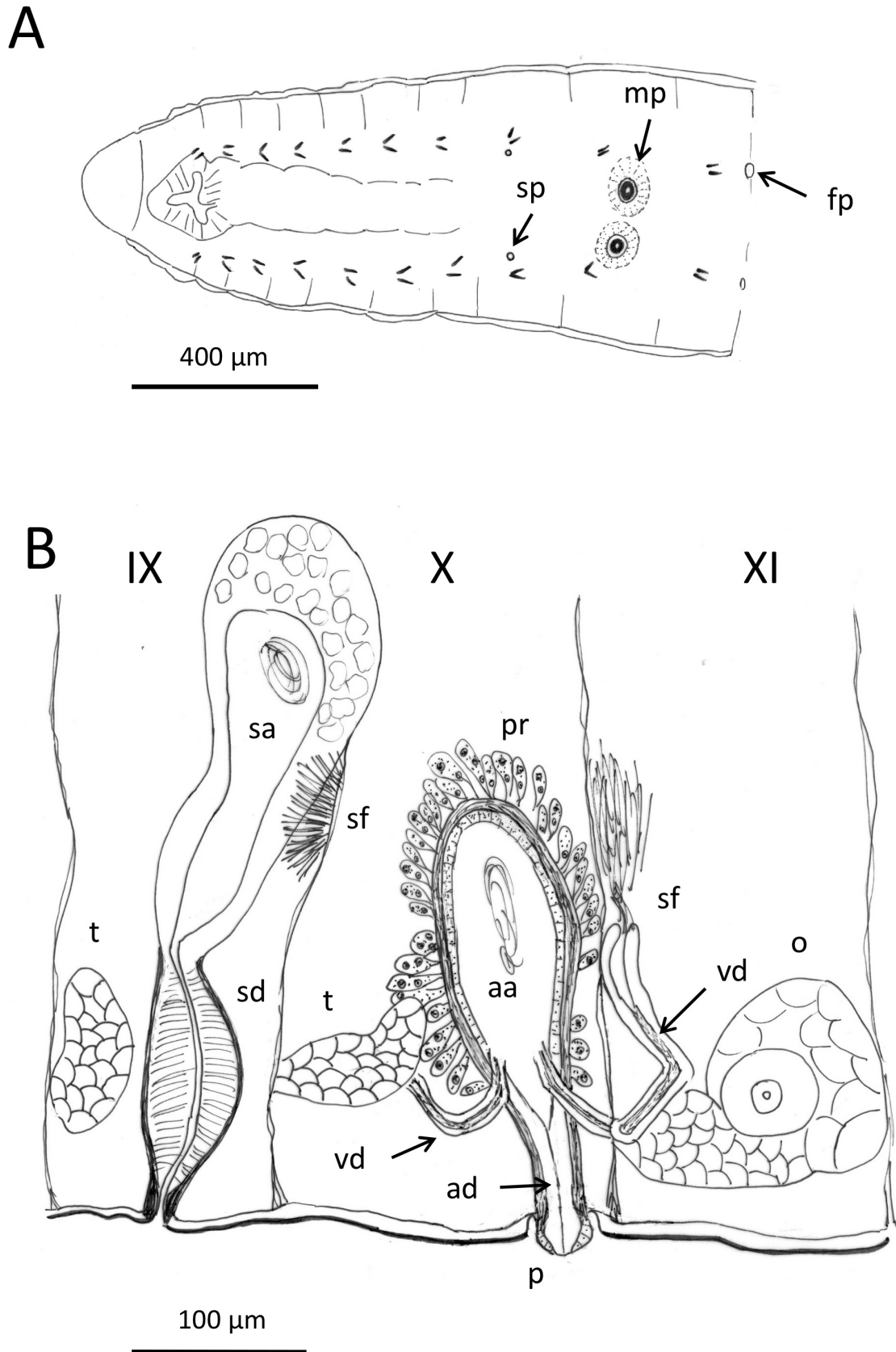


FIGURE 5. *Stylodrilus tofaceus* n. sp. **A.** Schematic drawing of the anterior body region, ventral view, showing spermathecal and male pores. **B.** Schema of the reproductive segments.

Clitellum from the line of chaetae in IX to the end of segment XIII, both dorsally and ventrally. One pair of male pores in X, behind ventral chaetae, opening closely to septum 10/11, and displaced medially from the line of ventral chaetae (Figs. 4E, 5A). One pair of spermathecal pores in IX, close to and medially of the ventral chaetae, on the transverse plane containing the chaetae (Figs. 4D, 5A). Female pores in the intersegment 11/12, in line with ventral chaetae.

Body wall formed by smooth epidermis (up to 17 μm high, to 27 μm at the clitellum), circular musculature (3–5 μm thick) and longitudinal musculature (16–20 μm thick dorsally and 27–50 μm ventrally). Pharynx with dorsal pharyngeal pad in II and III. Pharyngeal glands from IV to VI with dorsal, lateral and ventral lobes, and in VII only a small pair of ventral lobes. Chloragogen cells cover the gut from the posterior part of VI, backward (from V in one specimen). First nephridia at 6/7, and also observed at the ventral side of several postclitellar segments. Posterior lateral blood vessels with branches observed in only one specimen, not seen in the rest of material, probably due to bad fixation of posterior body section, which generally appears broken or beaded; thus, their presence and shape should be confirmed in new material.

Paired testes in IX and X, one pair of ovaries in XI. Sperm sac extending to VII anteriorly and to XIII posteriorly, ovisac to XIV. One pair of semi-prosoporous atria in X, two vasa deferentia per atrium, with male funnels at 9/10 and 10/11, both functional with sperm (Fig. 5B). Vasa deferentia 15–18 μm in diameter, the anterior one entirely in X, the posterior one entering XI where it forms a loop before joining atrium. The vasa deferentia join the atrium separately but closely to each other at the basal third of the ampulla, run toward the apical part of the atrium, and open at the basal part of the ampulla into the atrial lumen. Atrium elongate (198–267 μm long), club-shaped, with ectal end gradually narrowed towards pore, its length about one half of the body diameter in X. Atrial ampulla 141–155 μm long, 64–84 μm in diameter; atrial muscle layer 5–10 μm thick, epithelium 5–8 μm high, ampulla covered by a layer of prostatic cells organized in small clusters, up to 45 μm high (Fig. 4F). Atrial duct not well separated from the ampulla, devoid of prostatic cells, about 110 μm long, 27–34 μm in diameter, and covered by a muscle layer (3–5 μm thick). Atrium opens in a simple epithelial invagination, with a conical penis (17–25 μm long, 27–34 μm diameter) (Figs. 4G, 5B).

One pair of spermathecae in IX (Fig. 5B). Sac-like spermathecal ampulla (254 μm long and 90 μm wide) with strongly vacuolated epithelium in its ental region. Spermathecal duct spindle-shaped, 113–149 μm long, maximum diameter 58–88 μm (narrowed at both ends of the duct to 23–25 μm), shorter than ampulla, with a narrow lumen and lining epithelium 28–40 μm high at the widest section, surrounded by a thin muscle layer (c. 2 μm) which becomes thicker distally (to 6 μm) (Figs. 4H, 5B). Female funnels at 11/12.

Remarks. The new species is morphologically close to other *Stylodrilus* species with simple-pointed chaetae and elongate atria (tubular, cylindrical or elongated) (see Table 3). Among these species, *S. subcarpathicus* (Hrabě, 1929) (not included in Table 3) is clearly separated from any other known *Stylodrilus* species by the very long, tubular atrium which extends to segment XII. The species *S. glandulosus* Giani & Martínez-Ansemil, 1984 and *S. curvithecus* Collado, Martínez-Ansemil & Giani, 1993 are also well separated from other congeneric species by the presence of long atria that pass to segment XI, and the atrial duct associated with a bulb surrounding a penial sac, i.e. an invagination of the body wall containing the penis. *Stylodrilus* species reported in the Table 3 are all of medium size (≤ 1 mm body diameter) and share the following characters: prostatic cells usually organized into clusters; junction of vasa deferentia to atrial ampulla basal, and opening to atrial lumen medially to apically; penis short and/or conical (absent only in *S. sulci*). The new species is mainly distinguished from other *Stylodrilus* species by the relatively small size of the atrium, similar in size to the atrium in *S. coreyi* Rodríguez *et al.*, 2014 (see Table 3), the position of spermathecal pores in the transversal plane containing ventral chaetae and toward the ventral midline of the body, and the shape of the spermathecal duct, spindle-shaped, and formed by very high lining cells. Among *Stylodrilus* species known so far, spermathecal ducts are generally tubular, as in *S. sulci* (Fig. 4I) or tubular to barrel-shaped depending on the contraction of the muscular layer of the duct, as in *S. lemani* (Figs. 4J, K). Short, spindle-shaped spermathecal ducts with long lining cells were also described in *S. mollis* Timm, 1998, but this species has bifid chaetae and other differences in the structure of the atrium. Commonly, the spermathecal pores in *Stylodrilus* open behind the ventral chaetae and in line with them; the position of spermathecal pores in *S. tofaceus* n. sp. is similar to that in *S. lemani* (P. Rodríguez, personal observation).

Immatures in the same samples were ascribed to the species based on the form of the chaetae (simple pointed), position of pharyngeal glands (back to segment VII) and first nephridia (in 6/7), as well as the typical position of gonads when partially mature.

TABLE 3. Comparison between *Stylodrilus* species with simple-pointed chaetae and elongate atrium. Excluded here is *S. subcarpathicus* (Hrabě, 1929), a species with very long tubular atria. Abbreviations: L, length; Ø, diameter; ant, anterior; post, posterior; –, no data. All data from the original descriptions, except for: (1) from Rodriguez (1988); (2) atrium length calculated here as the sum of atrial ampulla and duct mean values; (3) measurements from specimens in Hrabě's collection in the Natural History Museum of Prague; (4) Hrabě (1970); (5) unpublished data from specimens in P. Rodriguez' collection; (6): Hrabě (1934).

	<i>S. chukotensis</i> Sokolskaya, 1975	<i>S. coreyi</i> Rodriguez <i>et al.</i> , 2014	<i>S. curvithesus</i> Collado <i>et al.</i> , 1993	<i>S. glandulosus</i> Giani & Martínez-Ansemil, 1984
Body diameter [mm]	0.9–1.0 in IX	to 0.8 in X–XII	0.3–0.4 in VI, to 0.7 in XVI	0.4 in VI
Chaetae length [µm]	110–115 (dorsals ≤ ventrals)	63–116 (dorsals = ventrals)	93–145 (dorsals < ventrals)	82–159 (dorsals < ventrals)
Pharyngeal glands	IV–V	IV–VIII	IV–VI	IV–VII
Lateral blood vessels	not seen	absent	absent	present
Atrium length [µm]	270 ⁽²⁾	176–390	300–402	460–660
Atrium length rel. to body Ø	c. 0.3	c. 0.5	> body Ø	> body Ø
Atrium position	–	in X, may pass to XI	in X, may pass to XI	in X and pass to XI (XII)
Atrial ampulla [µm]	L: 176–191 Ø: 88–91	L: 120–184 Ø: 43–70	L: 162–240 Ø: 103–140	L: ~ 400 (original Fig. 3) Ø: 120–151
Atrial duct [µm]	L ≤ 88 Ø: 22–44	L: ~40, in original Fig. 6) Ø: ~24	L: 76–168 Ø: 22–55	L: ~ 240 (original Fig 3) Ø: 42–45
Penis shape	small, conical	short	conical in a bulbous muscular penial sac	conical in a bulbous muscular penial sac
Penis length [µm]	–	25	39–71	84–92
Atrial musculature, thickness [µm]	9	4–6	8	–
Prostatic cells	forming clusters (Fig. 1.4 Sokolskaya)	forming clusters	prostate not well developed	forming a continuous layer
Vasa deferentia, junction with ampulla	basal junction, entrance at distal third of ampulla	basal junction, apical entrance	basal junction, subapical entrance	basal junction, subapical entrance
Vas deferens, diameter [µm]	18–22	20–28		
Post vas deferens enters s. XI	yes	no	no	no
Position of spermathecal pores rel. to ventral chaetae	behind, more ventrad than the line of ventral chaetae	behind, in line	behind, in line	behind, in line
Spermathecal ampulla	L: 420, Ø: 300–328	L: 205–348, Ø: 174–331	L: 448–673, Ø: 115–185	L: 186–232, Ø: 159–190
Spermathecal duct size (µm)	L: 209–228, Ø: 55	L: 150–247, Ø: 22–31	L: 148–246, Ø: 28–62	L: 195–355, Ø: 38–43
Spermathecal duct shape	–	thin, tubular	tubular (in original Fig. 1D)	tubular, widened at pore, with long glandular cells
Distribution (Habitat)	N.E. Russia (creek)	East USA (creek)	N.W. Spain (creek)	N.W. Spain (river)

TABLE 3. (Continued)

	<i>S. lemani</i> (Grube, 1879) ⁽¹⁾	<i>S. longiatriatus</i> Dembitsky, 1976	<i>S. sulci</i> (Hrabě, 1932) ⁽⁶⁾	<i>S. tofaceus</i> n. sp.
Body diameter [mm]	0.5–0.9 in IX–XIII	0.4–0.8	0.6	0.5 in X
Chaetae length [µm]	140–190	120–172	132–179 ⁽³⁾	80–132 (dorsals < ventrals; ant < post)
Pharyngeal glands	IV–VI	IV–VI	IV–VII ⁽³⁾	IV–VII
Lateral blood vessels	present	present	absent	not seen
Atrium length [µm]	329–447	743 ⁽²⁾	340 ⁽³⁾	198–267
Atrium length rel. to body Ø	< body Ø	> body Ø	c. 0.5 ⁽³⁾	c. 0.5
Atrium position	in X	in X–XI	in X	in X
Atrial ampulla [µm]	L: 329–447 (with duct) Ø: 85–91	L: 620–645 Ø: 129	L: 350 ⁽⁴⁾ , 210–217 ⁽³⁾ Ø: to 130 ⁽⁴⁾ [92–121 ⁽³⁾]	L: 141–155 Ø: 64–84
Atrial duct [µm]	Not distinctly separated	L: 114 Ø: 38–39	L: 106–121 ⁽³⁾ Ø: 27–38 ⁽³⁾	L: 110 Ø: 27–34
Penis shape	short	conical	absent	short, conical
Penis length [µm]	22–47	26	–	17–25
Atrial musculature, thickness [µm]	14–26	23	–	5–10
Prostatic cells	forming clusters	–	forming clusters ⁽³⁾	forming clusters
Vasa deferentia, junction with ampulla	basal junction entrance at distal third of ampulla	basal junction, subapical entrance	junction about middle	basal junction, middle entrance
Vas deferens, diameter [µm]	14–20	22–26	18–24 ⁽³⁾	15–18
Post vas deferens enters s. XI	yes	yes	yes (a little)	yes
Position of spermathecal pores rel. to ventral chaetae	behind, in line	lateral, toward body midventral line	behind, in line	lateral, toward body midventral line
Spermathecal ampulla	L: 282–472 Ø: 182–236	L: 600 Ø: 240	L: Ø: 181–236	L: 254 Ø: 90
Spermathecal duct size (µm)	L: 294–327, Ø: 57–83 ⁽⁵⁾	L: 314, Ø: 52–60	L: 140–200, Ø: 33–49	L: 113–149, Ø: 58–88
Spermathecal duct shape	tubular to barrel-shaped ⁽⁵⁾	–	tubular, widened at the pore ⁽³⁾	spindle-shaped
Distribution (Habitat)	South & Central Europe and Siberia (streams and springs)	Crimea (river)	Herzegovina (cave)	Croatia (tufa barrier)

Eclipidrilus sp.

(Figure 6)

Study material. 1 specimen from Roški slap, Croatia, 43°54'12.1"N , 15°58'31.3"E (43.90336 N, 15.975348 E) (November 6, 2013). Collector: Zlatko Mihaljević.

Description. Body consists of 61 segments, about 15 mm long. Prostomium short and pointed (250 µm long). Clitellum weakly developed, not raised, from the line of chaetae in IX to the line of chaetae in XIII. Secondary annuli not visible, but the specimen is very contracted due to fixation. Two chaetae per bundle, single pointed (Fig. 6A), 160–172 µm long, 7–8 µm thick in anterior segments, smaller in II and larger in VI, with the nodulus in distal position, at 0.3–0.4 distance from the tip. Chaetae shorter in postclitellar segments (146–151 µm), and slightly thinner (5–6 µm) than in anterior segments; in most posterior segments, chaetae 92–134 µm long, with nodulus at 0.3 distance from the tip. The ventral chaetae present and unmodified on clitellar segments. Spermathecal pores and male pores paired, surrounded by a ring of muscles, posterior to and in line with the ventral chaetae on IX and X, respectively. Female pores located in intersegment 11/12. Pharyngeal glands in segments IV to VI. Blind posterior lateral blood vessels not observed although the presence of large amounts of sediment particles in the gut makes the examination difficult. First nephridia observed in segments VII and VIII, and postclitellar from 12/13, backwards. Sperm sacs extending anteriorly to VIII and posteriorly to XV. Egg sac back to XV with eggs and yolk.

Paired testes in IX and X, ovaries paired in XI. One pair of ciliated female funnels on septum 11/12. One pair of atria in X, elongate (429 µm long, maximum diameter 97 µm) and entering segment XI; the transition of the atrial ampulla to the atrial duct is not abrupt. Atrial duct short, narrower than ampulla (88 µm long, 25–42 µm wide, thinner ectally), and devoid of prostate glands. The atrial ampulla consists of two muscle layers, the outer layer (10–12 µm thick) formed by muscular fibers that intersect in different directions and the inner layer (5–6 µm thick) arranged longitudinally; atrial epithelia 15–20 µm high. Atrial ampullae completely covered by prostate gland cells (up to 77 µm high), forming densely packed clusters (Fig. 6B). The atrium opens to the exterior through a pore located in a slightly elevated papilla, without a penis (Fig. 6C). Male duct semiprosoporous, with anterior sperm funnels not visible in the preparation; one pair of vasa deferentia per atrium (20–31 µm diameter), with a ciliated lumen, joining the atria in the basal part of the atrial ampulla, on opposite sides. The posterior vas deferens does not penetrate the ovarian segment.

One pair of voluminous spermathecae (about 750 µm long, 225 µm wide), bent, located in IX or extending into X. Ectal section of the ampullae with well-stained sperm, lining epithelium 15–18 µm high, not vacuolated; ental section of the ampullae with undetermined material and unstained sperm, lining epithelium up to 45 µm high (Fig. 6D). Spermathecal duct 300–350 µm long and 45–75 µm wide, covered by a thin muscle layer (4–7 µm thick), high lining cells (10–18 µm), and with a narrow lumen (4–9 µm) (Fig. 6E); the distal part covered by small glandular cells (Fig. 6F).

Remarks. We identify this specimen as a member of the genus *Eclipidrilus* Eisen due to the general organization of the reproductive system (semiprosoporous male duct and spermathecae in the first testicular segment), the elongate atrium and the presence of two muscular layers in the atrium, one of them with fibers arranged crosswise. Arrangement of the atrial musculature in the Croatian specimen differs from that of other *Eclipidrilus* species by the atrial musculature organization, since the crossed musculature is the outer layer while it is the inner layer in other species known so far (see amended diagnosis in Fend & Lenat 2012). Otherwise, the most similar *Eclipidrilus* species is *E. fontanus* Wassell, 1984 (atria paired, in X, no cuticular penis, no proboscis, paired spermathecae with simple ducts). Given the lack of more material for a detailed histological study of the male duct, this species cannot be attributed to *E. fontanus* or be described as a new one, since several characters, e.g. the presence of glands at the male and spermathecal pores and the structure of distal section of the atrial duct (= ejaculatory duct) must first be clarified. It is noteworthy that the genus *Eclipidrilus* appears to be a Nearctic genus and only *E. lacustris* has been reported on few occasions in Europe (Britain, Italy: Brinkhurst & Cook 1966; Cook 1971; The Netherlands: Verdon-schot 1990), possibly due to recent introductions. However, *E. lacustris* is easily distinguished from our specimen by single, median male and spermathecal pores, and by a distinct cross-hatched atrial musculature.

The general organization of the reproductive system in the genus *Eclipidrilus* is similar to that of the genus *Stylodrilus* Claparède, as described by Cook (1971), i.e. the male duct is semi-prosoporous, although it usually shows

a reduction in the anterior sperm funnel (Fend & Lenat 2012). In the examined specimen, the anterior sperm funnels were not observed, although the anterior vas deferens has similar diameter to the posterior one.

Within *Stylodrilus*, some species share this type of elongate, petiolate atria: *S. subcarpathicus*, *S. longiatratus* Dembitsky, 1976, *S. glandulosus* and *S. curvithecus*, all with simple-pointed chaetae and distributed in South and Central-East Europe. However, all of them have sperm funnels of similar size and the atrium has a simple organization of the musculature, in one layer. Only the species *S. subcarpathicus* has been formerly regarded as a possible member of the genus *Eclipidrilus*, due to its very long atria which extend backwards to XII (Cook 1971), but other generic characters such as the atrial musculature and the different size of anterior and posterior sperm funnels are not reported for that species, thus, they probably do not differ from the typical characters in the genus *Stylodrilus* (see discussion on these differences in Fend 2005).

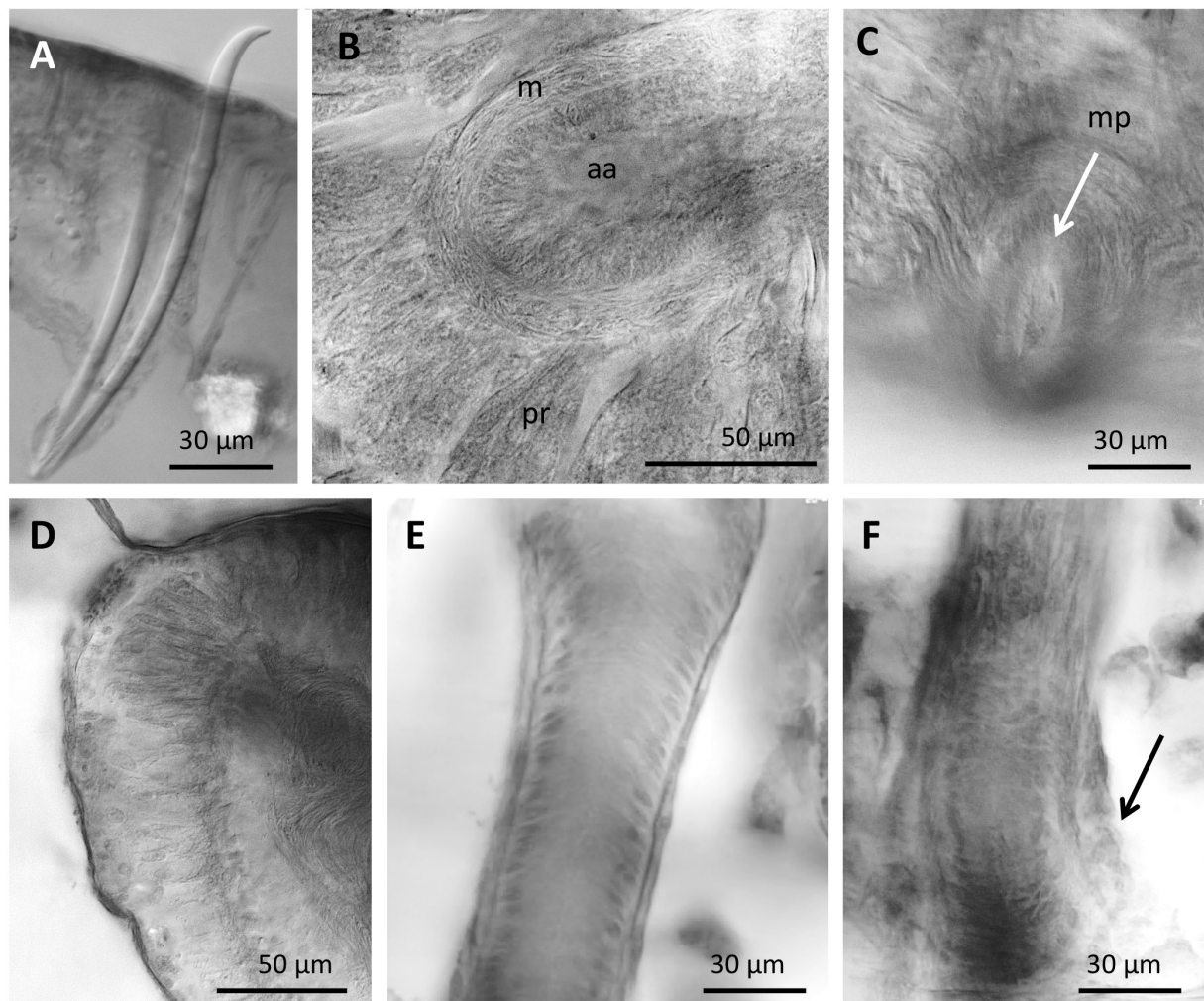


FIGURE 6. *Eclipidrilus* sp. **A.** Chaeta. **B.** Atrium showing prostate glands forming clusters of cells, and inner and outer layers of muscular fibers. **C.** Male pore. **D.** Ental part of spermathecal ampulla. **E.** Ental part of spermathecal duct showing high lining cells. **F.** Ectal part of spermathecal duct showing small glandular cells (arrow) near the pore.

Discussion

Prior to this study, the species list of oligochaete worms from tufa barriers of Croatia included only one lumbriculid species (*Stylodrilus heringianus* Claparède) and no phallodrilines, although both lumbriculids and phallodrilines are well represented in groundwaters of the region. The subterranean freshwaters in the Dinaric region have been intensely studied, and published records include 4 phallodriline species and 23 lumbriculid species (Martínez-Ansemil *et al.* 2016), most of them endemics. The new species from tufa barriers in karstic waters of Croatia are

members of the genera *Aberrantidrilus* and *Stylodrilus*, both present in groundwaters of southern Europe, a fact that probably reflects a connectivity of tufa barriers with groundwaters. Among other invertebrate taxa found in the same tufa samples, we can highlight the finding of *Gammarus balcanicus* Schäferna (Karaman 1977), an example of a typical karst freshwater amphipod that has been regarded as stygophile species, due to its presence in both surface and groundwaters (e.g. Pacioglu *et al.* 2019). These findings also support future study efforts on tufa habitats, which may increase the knowledge of the biodiversity of both phallodrilines and lumbriculids, and provide tools for conservation policies.

Twenty-three freshwater phallodriline species have been described so far, most of them from southern Europe (Appendix 1). Among them, 18 have been reported in different groundwater habitats (springs, cave rivers, hyporheic and phreatic waters). The remaining species have been reported from rivers, pools, or lake sediments, although they could be regarded as members of the interstitial fauna, due to their small body size. The finding of *A. mihaljevici* n.sp. suggests the existence of a colonization route between tufa barrier habitats and groundwaters. It also contributes to a more complete picture on the evolutionary relationships within *Aberrantidrilus*, a genus distributed throughout southern Europe in a variety of groundwater habitats and tufa, the new species being morphologically closer to congeners in Slovenia and Italy (*A. cuspis*) than to other species in France (*A. stephaniae*) or Spain (*A. subterraneus*).

The description of the new species increases the number of aquatic oligochaete species known in Croatia to 73 (see Kerovec *et al.* 2016), and gives a first record of a phallodriline species in Croatian freshwaters [*Spiridion modricensis* (Hrabě, 1973) was reported in Croatia from a salt spring, see Appendix 1]. This study also stresses the importance of exploring new habitats to get a better picture of the European biodiversity in freshwaters, where there are still species to discover. At the same time, the new findings focus interest on the study of oligochaete communities in tufa barriers, as well as on the possible interactions between this interesting habitat and groundwaters.

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APPENDIX 1. Phallodrilinae species reported in freshwater habitats. Total number of genera: 8. Total number of species 23.

Aberrantidrilus Martin, 2015

A. cuspis (Erséus & Dumnicka, 1988). Subterranean waters in wells from several localities in Perugia, Italy. Other reports: Martin *et al.* 2015: Subterranean waters in Italy, France, Slovenia.

A. subterraneus (Rodriguez & Giani, 1989). River into Cave Jivero-1, Cantabria, Spain. Other reports: Achurra *et al.* 2015: in type locality.

A. stephaniae Martin, 2015. Hyporheic habitats in Mercantour National Park, France.

A. mihaljevici Vučković, Rodriguez & Kerovek **n. sp.** Tufa in Roski slap, Croatia.

Aktedrilus Knöllner, 1935

A. argatxae Giani & Rodriguez, 1988. Spring of Argatxa cave, Spain. Other reports: Achurra *et al.* 2015: several localities in subterranean waters of North Spain.

A. leeuwinensis Pinder, 2006. Pool area of a stream within Budjur Mar Cave, Australia.

A. monospermathecus Knöllner, 1935. In groundwater and interstitial water in several localities of Kiel Bay, and close to Schleswig, Germany; water of variable salinity: 0–18 ppt (after Hrabě 1960). Other reports: Hrabě 1960: in river Weser, Bremen, Germany; Giere 1977: osmoregulator interstitial worm, tolerant to salinity, optimal salinity in Baltic Sea ca. 10 ppt; Martinez-Ansemil 1990: River Tambre, Spain, 0–7.5 ppt.

A. podeilema Pinder, 2006. Sand and fine black silt with organic matter in a deep clear pool of freshwater. Tombstone Rocks Cave, Nambung region, Western Australia.

A. ruffoi Sambugar, Giani & Martínez-Ansemil, 1999. Near the spring of the river Tione, Verona, Italy.

A. yacoubii Martin & Boughrous, 2012. Groundwater from Ain Maarouf exsurgence, Morocco.

Coralliodrilus Erséus, 1979

Coralliodrilus amissus Arslan, Timm & Erséus, 2007. Göksu Stream, Turkey.

Gianius Erséus, 1992

Gianius anatolicus Arslan, Timm & Erséus, 2007. Göksu Stream, Turkey.

Gianius aquaedulcis (Hrabě, 1960). Lower river Weser, Germany. Other reports: Rodriguez & Giani 1989: river in Jivero-1 Cave, Spain; Farara & Erséus 1991: river Niagara, USA; Dumnicka 2009: karst springs in Poland; Achurra *et al.* 2015: several localities in subterranean waters of North Spain.

Gianius cavealis Juget & des Chatelliers, 2001. Groundwaters in Mongelas karst complex, France.

Gianius crypticus (Rodriguez & Giani, 1989). Cave del Orillon, Santander, Spain.

Gianius labouichensis (Rodriguez & Giani, 1989). River in cave Labouiche, France.

Gianius navarroii Rodriguez & Achurra, 2010. Stream in cave Ojo Guareña, Spain.

Gianius riparius (Giani & Martinez-Ansemil, 1981). River Tambre, Spain. Other reports: Lafont & Durbec 1990: groundwater of Grand Gravier, river Rhone, France.

Pectinodrilus Erséus, 1992

Pectinodrilus ningaloo Pinder, 2006. Groundwater in a well, Australia.

Phallobaikalus Martínez-Ansemil, 2010 (in Martin *et al.* 2010)

Phallobaikalus gladiiseta (Martin & Brinkhurst, 1998). Lake Baikal, 310 and 580 m depth.

Spiridion Erséus, 1992

S. insigne Knöllner, 1935. Hrabě 1960: Groundwater in Weser river, Blumenthal, Germany.

S. phreaticola (Juget, 1987). Hyporheic and phreatic groundwaters in alluvial plain of river Rhône, France. Other reports: Route *et al.* 2004: Hyporheos of Lachein Creek, France.

Note: The species *S. modricensis* (Hrabě, 1973) is not considered here, since it was found in a salt spring in Croatia, close to the sea.

Thalassodrilus Brinkhurst, 1963

Thalassodrilus hallae (Cook & Hiltunen, 1975). Freshwater, sublittoral and profundal sediments, Lake Superior.