Zootaxa 2332: 21–39 (2010)
www.mapress.com/zootaxa/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

New species of aquatic oligochaetes (Annelida: Clitellata) from groundwaters in karstic areas of northern Spain, with taxonomic remarks on *Lophochaeta ignota* Štolc, 1886

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Abstract

We describe two new species: *Gianius navarroi* **n. sp.** (Phallodrilinae) and *Isochaetides gianii* **n. sp.** (Tubificinae), which were discovered during investigations on the groundwater oligochaete fauna in northern Spain. The present study contributes to the knowledge of the stygobiont oligochaete species in the Iberian Peninsula, which includes 21 species so far. Mature specimens of *Lophochaeta ignota* Štolc, 1886 were also collected at some sites, and are used to supplement the limited description of the reproductive organs known for the species to date. Taxonomy of the genus *Lophochaeta* Štolc, 1886 is discussed.

Key words: Oligochaeta**,** *Gianius, Isochaetides, Lamadrilus*, stygobiont, taxonomy

Introduction

The present contribution is part of a more extensive study on the groundwater oligochaetes from several karstic units in the Cantabric region and the Basque Country, in northern Iberian Peninsula (Spain). The study area is situated within a hypothetical band (between *ca* 42º N and 46º N in Europe) of high biodiversity for terrestrial cave fauna (Culver *et al*. 2006), based on historical (mostly climatic) conditions. Consequently, we also expect a high biodiversity for the aquatic fauna.

Two recent contributions have presented data on the groundwater oligochaetes from northern Spain (Camacho *et al.* 2006; Achurra & Rodriguez 2008). The Ojo Guareña cave system has produced 15 taxa, including a new undescribed Phallodrilinae. The cave is one of the largest karstic systems in Europe (*ca* 60 km explored), and several sampling surveys of epikarstic groundwater fauna were organized by the National Museum of Natural Sciences (Madrid), to study the distribution patterns of subterranean invertebrates in different habitats of the cave (Camacho *et al.* 2006). The karst of Santa Eufemia–Ereñozar was reported to have 47 taxa, including an undetermined Tubificinae. The karst is mostly located within the Urdaibai Biosphere Reserve, it was investigated during two independent sampling surveys (1983–86 and 2005–06), and was suggested to be a "hotspot" of biodiversity for groundwater oligochaetes (Achurra & Rodriguez 2008).

This paper contributes to the knowledge of biodiversity in groundwaters with the description of two new species from the above mentioned collections: *Gianius navarroi* **n. sp.** (Phallodrilinae) and *Isochaetides gianii* **n. sp.** (Tubificinae). Additional data on some morphological features, as well as a detailed description of the reproductive organs of the species *Lophochaeta ignota* Štolc, 1886 are based on mature specimens from sites in the Santa Eufemia–Ereñozar karst unit. Most published records of that species in running waters are based on immature specimens, identified on the basis of their thin outline and chaetal features, and mature individuals have been described on few occasions (Štolc 1886, 1888; Hrabě 1962, 1981; Laakso 1969;

Accepted by B. Sket: 10 Nov. 2009; published: 11 Jan. 2010

Chekanovskaya 1981). Thus, measurements of the reproductive organs in *L. ignota* are scarce; and our description aims to contribute not only to the knowledge of the male duct characteristics but also to clarify the taxonomy of this species.

Material and methods

Different collecting methods were used in this study, including Surber nets and hand-nets (100 and 200 µm mesh size). The specimens from the Ojo Guareña cave system (Burgos, Spain) were found during two sampling surveys (2002–2004 and 2007) by the National Museum of Natural Sciences (MNCN), in several aquatic habitats (streams, gours and puddles). The specimens from the karst of Santa Eufemia–Ereñozar (Biscay, Spain) were collected in springs and caves, during two independent sampling surveys (1983–86 and 2005–06).

Samples were fixed in the field with 4–10% formaldehyde and washed through a 100 µm sieve before sorting under a stereo-microscope at the laboratory. Then, specimens were preserved in 70% ethanol and oligochaetes identified using a Nomarski Interference Contrast microscope. Worms were whole-mounted in glycerol or in lactophenol. Some specimens were permanently prepared in Canada balsam, either whole-mounted or dissected after staining with Ehrlich´s hematoxylin, dehydration and clearing in creosote. Histological sections (5 µm) were stained with hematoxylin and eosin, and mounted in DPX.

The type specimens are maintained in the National Museum of Natural Sciences of Madrid, Spain (MNCN), the National Museum of Natural History (Smithsonian Institution) of Washington, DC (USNM) and in the collection at the University of the Basque Country (UPV/EHU).

For comparison, we have studied the following additional material, loaned by the USNM and Dr. Tarmo Timm (Estonia):

Isochaetides freyi (Brinkhurst, 1965): Holotype: USNM 32628, Paratypes: USNM 32629

Lamadrilus sorosi Timm, 1998: Paratypes 22-3, 22-4, 22-7 and other material, both whole-mounted (2 individuals) and sectioned (3 individuals), loaned by Dr. Timm.

Lophochaeta ignota Štolc, 1886: several specimens in 70% alcohol from Aheru järv (lake in South Estonia), 18 July 1989; and from Varzuga (river on the Kola Peninsula, Murmansk Region, Russia), 28 July 1969, from Dr. Timm's collection.

Results

***Gianius navarroi* n. sp.**
(Figures 1–2)

Phallodrilinae **n. sp.** in Camacho *et al.* 2006.

Holotype. MNCN 16.03/3051. An incomplete mature specimen, stained in Ehrlich's Haematoxylin and whole-mounted in Canada balsam.

Type locality. Ojo Guareña cave (site: "Granja Ortiz", 1 June 2002), northern Burgos, Spain. UTM coordinates for the Palomera entrance: X:446595, Y:4764790, Z:724.

Paratypes. MNCN 16.03/3052. A complete mature specimen (unmated) from the type locality, site: "Gour de las Hojas" (8 March 2003). A complete mature specimen in the collection at the University of the Basque Country (UPV/EHU), from the type locality, 11 June 2007. Both specimens are stained in Ehrlich's Haematoxylin and whole-mounted in Canada balsam.

Etymology. Named after the physiologist Dr. Enrique Navarro who has helped P. Rodriguez in sampling numerous sites for the study of aquatic oligochaetes, and has collaborated in applied research with oligochaetes.

Description. Length of preserved worms 1.7 mm; number of segments (in two complete specimens) 23 and 26; maximum diameter 150 µm in segment XI. Prostomium rounded, with glandular epidermis (Fig. 1A). Pygidium short and wide (Fig. 1B). Body wall consisting of a fine cuticule (*ca* 1 µm thick), an epidermal layer (5 µm thick) and a relatively poorly developed musculature (2–3 µm thick). Clitellum formed by glands disposed in one layer, from the last third of segment X back to the beginning of segment XII. There is no secondary annulation. One pair of simple spermathecal pores in the most anterior part of segment X, opening in line with ventral chaetae. One pair of male pores in segment XI on porophores, in line with ventral chaetae. All chaetae bifid, 24–27 µm long, with distal tooth shorter than proximal. Anterior segments with 2–5 chaetae per bundle, 2 in postclitellar segments. Chaetal bundles are in the posterior third of the segment. A bundle of 5 penial chaetae forms an arc, towards the sagittal plane of the body (Fig. 2). Penial chaetae straight, with bent distal end, bifid, and of similar length to somatic chaetae.

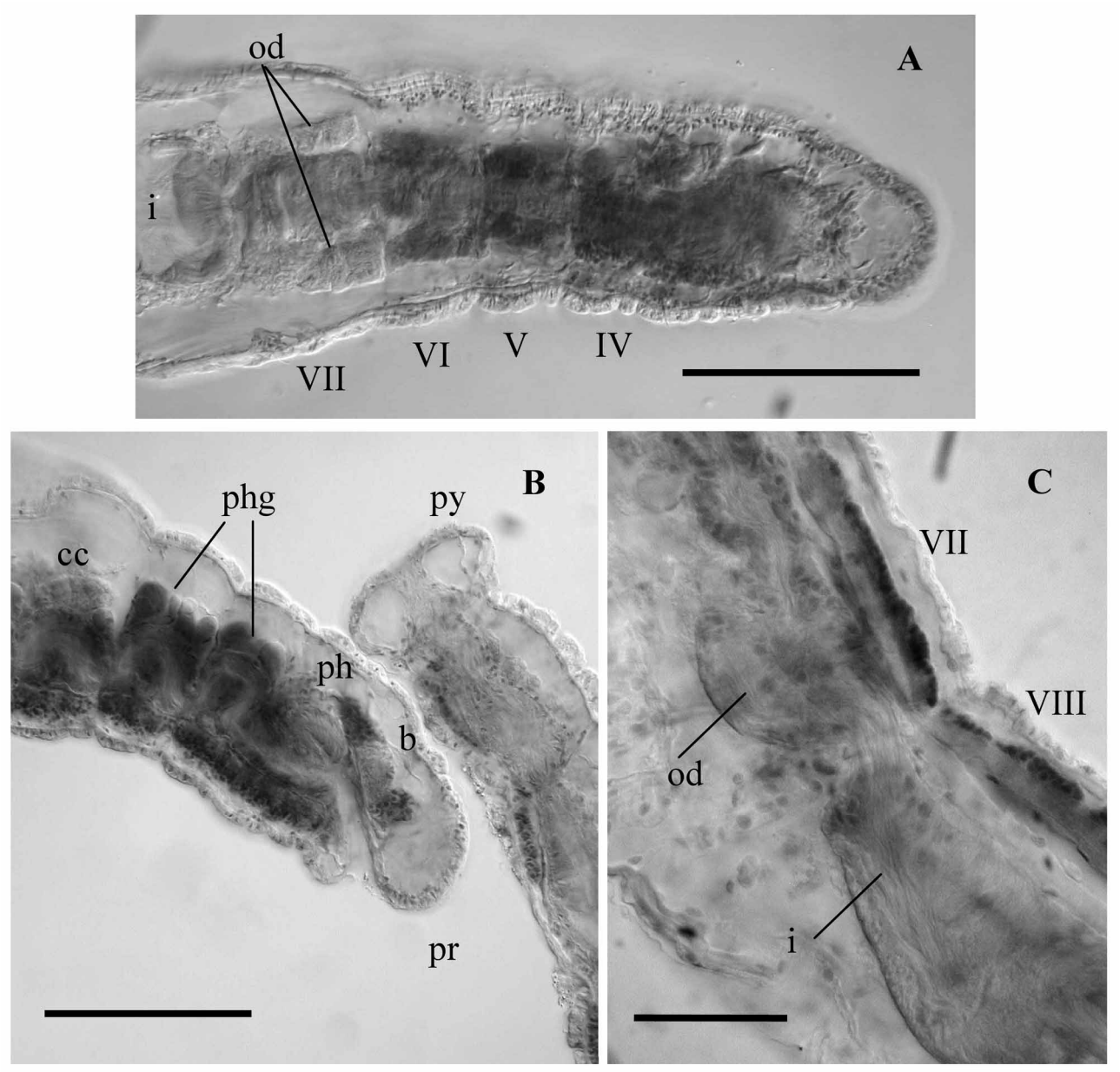

FIGURE 1. *Gianius navarroi* **n. sp.** A: anterior part of the body in dorso-ventral view, IV–VII: segment number; B: anterior and posterior ends of the body in lateral view; C: segments VII and VIII. **Abbreviations**: b: brain, cc: chloragogen cells, i: intestine, od: oesophageal diverticula, ph: pharynx, phg: pharyngeal glands, py: pygidium. **Scale bars**: A and B: 100 µm, C: 50 µm.

Brain with two histologically distinct sections: anterior and posterior parts having large nuclei intensely stained with haematoxylin, and a granulated median part that does not stain well. No coelomocytes observed within the coelomic cavity. Pharyngeal glands in segments III–V, dorsal in III, and both lateral and ventral in segments IV and V (in a paratype, glands also ventrally in VI). Chloragogen cells form a layer on the gut from segment VI backwards. In segment VII, a pair of dorsal digestive diverticula, can occupy half or the entire length of the segment, and appear to be attached to the oesophagus at the posterior end of the segment (Figs. 1A, C). A dilatation of the gut in segment VIII is interpreted as the beginning of the intestine (Figs. 1B, C).

One pair testes in segment X and one pair ovaries in segment XI. Sperm sacs in segment XI (in one specimen spermatogonia and spermatocytes are free in the coelom of segment X). Male ducts paired, with two prostate glands: one ental, close to the junction of the vas deferens with atrium; and other ectal, close to the male pore, much smaller than the ental one. A ciliated vas deferens (6 µm diameter) joins the atrium apically. Atria tubular (77 µm long, up to 15 µm diameter) and curved; thicker in the middle in lateral view (with homogeneous diameter in dorsal view) and gradually narrowing to both ental and ectal ends. The atrial musculature is thin (2–3 µm thick) and the lumen is ciliated or filled with granules (as in Fig. 2). The atria open into salient round porophores (*ca* 10 µm long), where the penial chaetae are located. Female funnels were not observed.

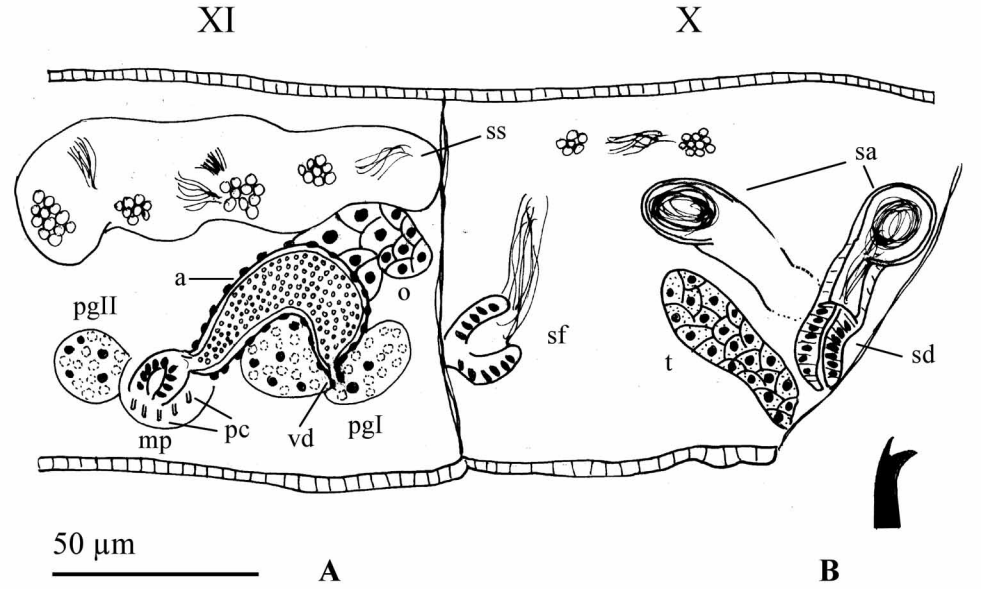

FIGURE 2. *Gianius navarroi* **n. sp.** A: general diagram of the reproductive organs of the species, X and XI: segment number; B: free-hand drawing of somatic chaeta. **Abbreviations**: a: atrium, mp: male pore, o: ovary, pc: penial chaetae, pgI and pgII: prostate glands I and II, sf: sperm funnel, sa: spermathecal ampulla, sd: spemathecal duct, ss: sperm sacs, t: testis, vd: vas deferens.

One pair of spermathecae, with a stout duct (20 µm long, 13–16 µm wide) and an elongated globular ampulla (40–50 µm long, 16–17 µm maximum diameter), which contains sperm forming a loop.

Distribution and habitat. *Gianius navarroi* **n. sp.** is known only from Ojo Guareña cave system, and it is likely to be a stygobiont. The species was collected from two different epikarstic sites within the cave, but was rare, at most one specimen per sample. Habitats sampled were puddles and cave streams, having temporary or permanent water, at depths up to 40 cm; sediment was composed of mud, sand and/or stones. Water temperature was 8.4–9.9ºC, pH 7.8–8.0, dissolved oxygen 74.3–78.4 % saturation and conductivity 389–465 µS/cm.

Taxonomic remarks. *Gianius navarroi* **n. sp.** has spermathecal pores in line with the ventral chaetae, spermathecae with short ducts, vas deferens entering the apical end of an elongated and curved atrium, and two prostate glands per atrium attached to proximal and distal ends, respectively. All of these characteristics are diagnostic for the phallodriline genus *Gianius* Erséus, 1992. The presence of digestive diverticula in

segment VII was previously known only in the species *Gianius labouichensis* (Rodriguez and Giani, 1989); although the connection of the diverticula with the gut appears to be in the posterior end of the segment for the new species, whereas for *G. labouchensis* the single diverticulum connects anteriorly. However, the latter is a distinctive species, well distinguished from other congeners by the lack of penial chaetae, a uniformly tubular atrium with a protrusible pseudopenis, and elongated narrow spermathecal ampulla. The general structure of the male duct in *G. navarroi* **n. sp.** is more similar to the remaining species of the genus, being closest to *G. aquaedulcis* (Hrabě, 1960), *G. cavealis* Juget and des Chatelliers, 2001 and *G. anatolicus* Arslan, Timm and Erséus, 2007 based on the thin atrial musculature and the moderate (4–5) number of penial chaetae. *Gianius cavealis* is well distinguished from the new species by its distal atrial duct well separated from an atrial ampulla, and *G. anatolicus* by the presence of a long, simple spermathecal duct and three different types of somatic chaetae. The general structure of male duct and spermatheca are similar to *G. aquaedulcis*, and differences are mainly related to the relative size of the prostate glands (both glands large and of about equal size, in *G. aquaedulcis* but of visibly different size in the new species), as well as the size of chaetae (41 µm in *G. aquaedulcis*) and the presence of oesophageal digestive diverticula. The *G. aquaedulcis* material described by Farara and Erséus (1991) in North America has a larger body size and wider atria (33–38 µm width); it is distinguished from the new species by the absence of noticeable porophores at the male pores, (described as "simple male pore on inconspicuous papilla"), and by the blunt ental end of the atria. The shape of the pygidium in *G. navarroi* is unusual, but very similar to that described for *Mexidrilus obtusus* by Erséus (1992: Fig.13).

Gianius navarroi **n. sp.** is separated from the previously known species of the genus by the presence of oesophageal diverticula, salient round porophores with the penial chaetae, and marked difference in size of prostate glands (ental gland larger than distal).

***Isochaetides gianii* n. sp.**
(Figures 3–5)

Lamadrilus sp. in Achurra and Rodriguez (2008)

Holotype. MNCN 16.03/3053. A dissected worm, stained in Ehrlich's Haematoxylin and mounted in Canada balsam.

Type locality. Apraiz karstic spring (6 September 2005), Santa Eufemia–Ereñozar karst unit, Biscay, Spain. UTM coordinates X:524225, Y:4801350, Z: 22.

Paratypes. USNM 2052660. A dissected specimen from the type locality (6 September 2005). MNCN 16.03/3054: two whole-mounted specimens from Argatxa spring (30 September 2005). In the collection at the University of the Basque Country (UPV/EHU): a sectioned and a dissected specimen from the type locality (6 September 2005); a dissected specimen from Oxiña spring (1 February 1985). All sites in Santa Eufemia–Ereñozar karstic unit, Biscay, Spain.

Other material. Three specimens preserved in 70% alcohol from the type locality (6 September 2005). Two dissected and three specimens in 70% alcohol, from Argatxa spring (30 September 2005). Four sectioned, seven dissected, two whole-mounted and 17 specimens preserved in 70% alcohol, from Aulestia spring (23 February 1985). All in the collection at the University of the Basque Country (UPV/EHU).

Other localities. Argatxa spring: UTM coordinates X:527790, Y:4800925, Z:2; Oxiña spring: X:529825, Y:4801300, Z:38; Aulestia spring: X: 536494, Y: 4794430, Z:66. All in Santa Eufemia–Ereñozar karst unit.

Etymology. Named after Dr. Narcisse Giani for his important contribution to the taxonomy and general biology of aquatic oligochaetes and also for his important role in the formation of many oligochaete taxonomists (including the first author).

Description. Body length 8–10 mm; segment number 59–83; mean body diameter 260 µm (range: 180–335 µm) in segment V, and 324 µm (225–390 µm) in segment VIII. Prostomium rounded or conical, 75–125

µm long, width about the same or wider at its base (all measurements in fixed individuals). Body wall consisting of a thin cuticle (<1 µm), smooth epidermis (5–10 µm thick, but up to 22 µm in the clitellum) and relatively poorly developed musculature (5–7 µm thick). There is no secondary annulation. Clitellum formed by a single layer of glandular cells, extended from anterior part of XI, back to the line of chaetae in segment XII; no glands in the region between the male pores. One pair of deep folds hiding spermathecal apertures (irregular in shape) in segment X, in line with ventral chaetae. One pair of male pores in segment XI, close to and slightly lateral to the ventral chaetae. In some specimens, the ventral part of segment XI appears deeply retracted by the action of dorso-ventral muscle strands.

Ventral chaetae 4–6 per bundle, reduced to two in posterior part of the body, 50–75 µm long, with distal tooth thinner and shorter than proximal (Fig. 3A, B). Hair and pectinate chaetae in dorsal bundles (Fig. 3C–E). Hair chaetae in preclitellar region 1–3 (seldom 4) per bundle, 162–262 µm long; in postclitellar region 1–2 per bundle, 75–125 µm long, gradually becoming shorter backwards. Pectinate chaetae in preclitellar segments 2–4 per bundle, 47–90 µm long and with distal nodulus (at about ⅓ to ¼ from the distal end); 2–3 somewhat shorter chaetae, posteriorly. Teeth of pectinate chaetae about equally long in anterior segments, but distal tooth shorter and thinner than proximal in posterior segments. The number of intermediate teeth in pectinate chaetae 1 or (usually) 2 in anterior dorsal chaetae; intermediate teeth are absent in posterior segments.

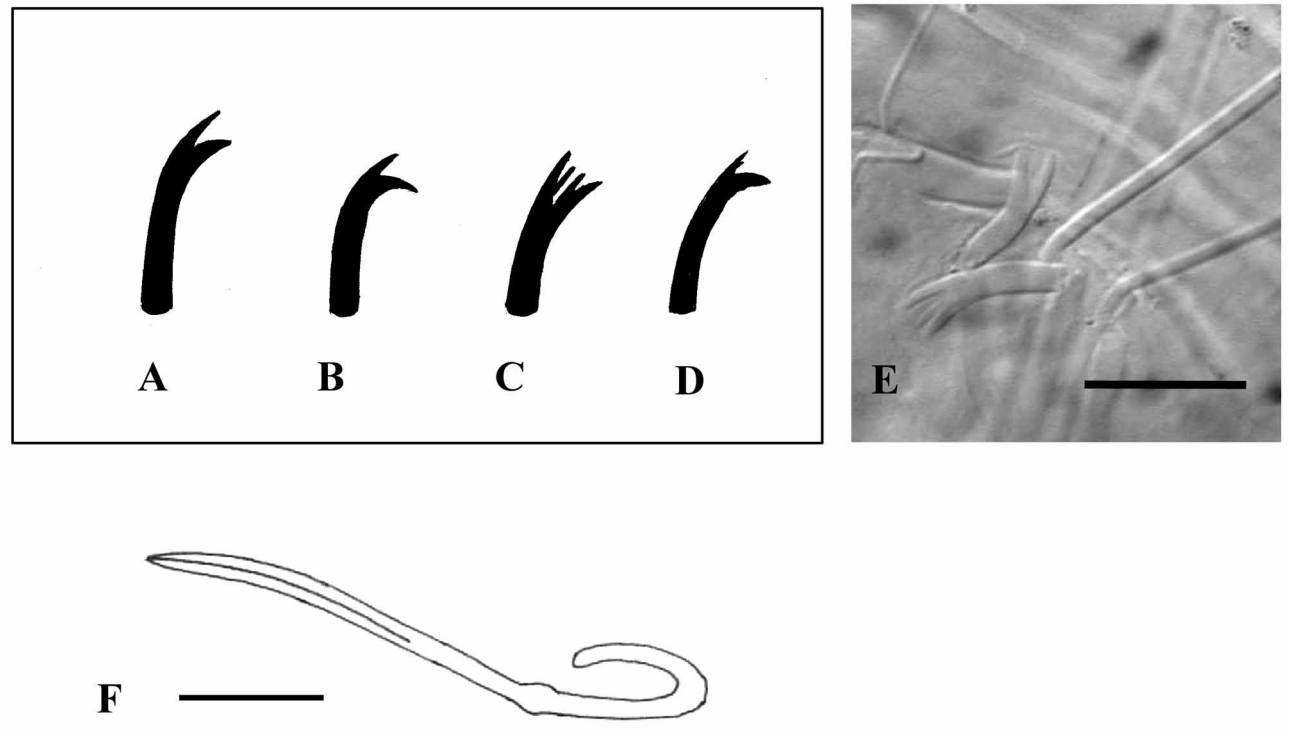

FIGURE 3. *Isochaetides gianii* **n. sp.** Somatic chaetae (A–D: free-hand drawings at the same scale). A: anterior ventral; B: posterior ventral; C: anterior pectinate dorsal; D: posterior dorsal; E: anterior bundle of pectinate and hair chaetae; F: spermathecal chaeta. **Scale bars**: E and F: 25 µm.

Modified, distally grooved spermathecal chaetae in segment X, one per bundle, 102–145 µm long and 2–3 µm thick (Fig. 3F). There are no modified penial chaetae; 1–2 somatic ventral chaetae per bundle, 50–52 µm long, are found ventrally in segment XI, lateral to the male pores.

Brain extends back to 1/2. Coelomocytes absent or, if present, they are scarce, small, oval (5 µm maximum diameter), nucleated and granular. Commissural blood vessels connecting dorsal and ventral vessels are visible in most examined specimens; they form several loops in anterior segments, particularly in V (Fig. 4A). Typical oligochaete dorsal, muscular pharyngeal pad not developed. In segments II–III, pharynx

with well-developed epithelium both dorsal and ventrally, and a large mass of glands dorsally (Fig. 4B). A few clusters of pharingeal glands are located dorso-laterally to the digestive tract in segment IV and laterally in V. A layer of chloragogen cells on the gut extends from segment VI backwards. In segment VIII, oesophagus opens abruptly into the intestine (Fig. 4C).

One pair of spermathecae in segment X. A short spermathecal duct (30–50 µm long, 30–50 µm wide), with a thick epithelium, opens ventrally in segment X at the inner end of a deep epidermal fold (Fig. 4D, E). Spherical to globular spermathecal ampulla (175–220 µm maximum diameter), with a thin epithelium (3–7 µm high, although up to 20 µm close to the connection with the spermathecal duct). The ampulla usually contains 4–5 drop-like, elongate spermatozeugmata (125–202 µm long, 35–82 µm maximum diameter), which consist of an external clear matrix 7–15 µm thick around an axial cylinder of longitudinally arranged fertilizing sperm (Fig. 4D). The spermathecal chaetae are orientated with their distal ends towards the spermathecal fold. The proximal end of the chaeta is bent, and associated with a chaetal gland (follicular cells) with several muscular strands; the distal end (*ca* one third of its total length) is grooved and contained within a chaetophore (32–50 µm diameter), which is internally glandular, radially arranged and externally surrounded by a muscular wall about 2–3 µm thick (Fig. 5). Close to the chaetophore, and most likely functionally associated with it, 1–3 accessory glands can be observed, with histological structure similar to that of the prostate gland. The position of the chaetophore is usually anterior to the spermathecal fold, although in some cases it can be posterior to the fold.

One pair testes in X and one pair ovaries in XI. Sperm sacs may extend to IX anteriorly and to XII posteriorly. Egg sac can reach segment XIII. Sperm funnel 80–127 µm high in lateral view (about one third the diameter of the body), attached to septum 10/11. Vas deferens ciliated throughout, two to three times longer than the atrium and penis, and restricted to segment XI, where it forms a few loops. It is up to 30 µm in diameter, but thinner in its proximal end (down to 13 µm diameter), and in some specimens also thinner just before its junction with the atrium (15 µm diameter). Atria in XI, tubular (250–300 µm long), bent and expanded near proximal end (42–60 µm, measured at the level of junction with the prostate), thinner in their distal part (30–40 µm), and swelling before entering the penial sac (Fig. 5). Distal section of atrium may be bent, forming a loop before entering the penial sac. Two different types of atrial cells, one forming a dense layer (up to 37 µm high) at the concave side of the atrium, and the other type with a clear granulated appearance forming a layer of cells (up of 20 µm high) along the convex side and the proximal part of the atrium. Atrial musculature very thin (<2 µm thick) and atrial lumen narrow, 2–5 µm. A medium-size, compact prostate gland connects by a short stalk to the concave side, at the proximal expanded end of the atrium. Vas deferens enters subapically on the convex side of the atrium. Penial sac well developed, oval, with maximum diameter parallel to the longitudinal axis of the body (115–145 µm), having a ring fold at its inner end. Penis cylindrical (65–115 µm long and 35–50 µm diameter) and formed by two epithelial layers (Fig. 4F–H). A mushroom-shaped cuticular layer, consisting of an inner oval part (2–4 µm thick) lining both the penial sac and the proximal ring fold; and a central stalk (1–2 µm thick) forming a cuticular sheath around the penis. This penial sheath is slightly conical and encloses most of the penis, which is free inside it and can project partially outwards. Female funnels were not observed.

Teratology. One specimen with two male ducts plus one spermatheca on one side, and one male duct, one spermatheca plus two chaetophores on the other side.

Distribution and habitat. The new species was found in four karstic springs located in the karst of Santa Eufemia-Ereñozar, in sandy sediments. Water characteristics were for Argatxa spring: 13.7ºC, pH 7.2, 446 µS/cm and 91 % dissolved oxygen; and for Apraiz spring: 14.8ºC, pH 7.0, 469 µS/cm and 70 % dissolved oxygen.

Taxonomic remarks. The position and general structure of the gonads, male ducts and spermathecae in the new species are characteristic of the oligochaetes of the subfamily Tubificinae. The new species is related to the tubificine genera *Isochaetides* Hrabě, 1966 and *Lamadrilus* Timm, 1998 by the presence of a tubular atrium, a discrete prostate gland attached to atrium by a narrow stalk, a vas deferens longer than the atrium

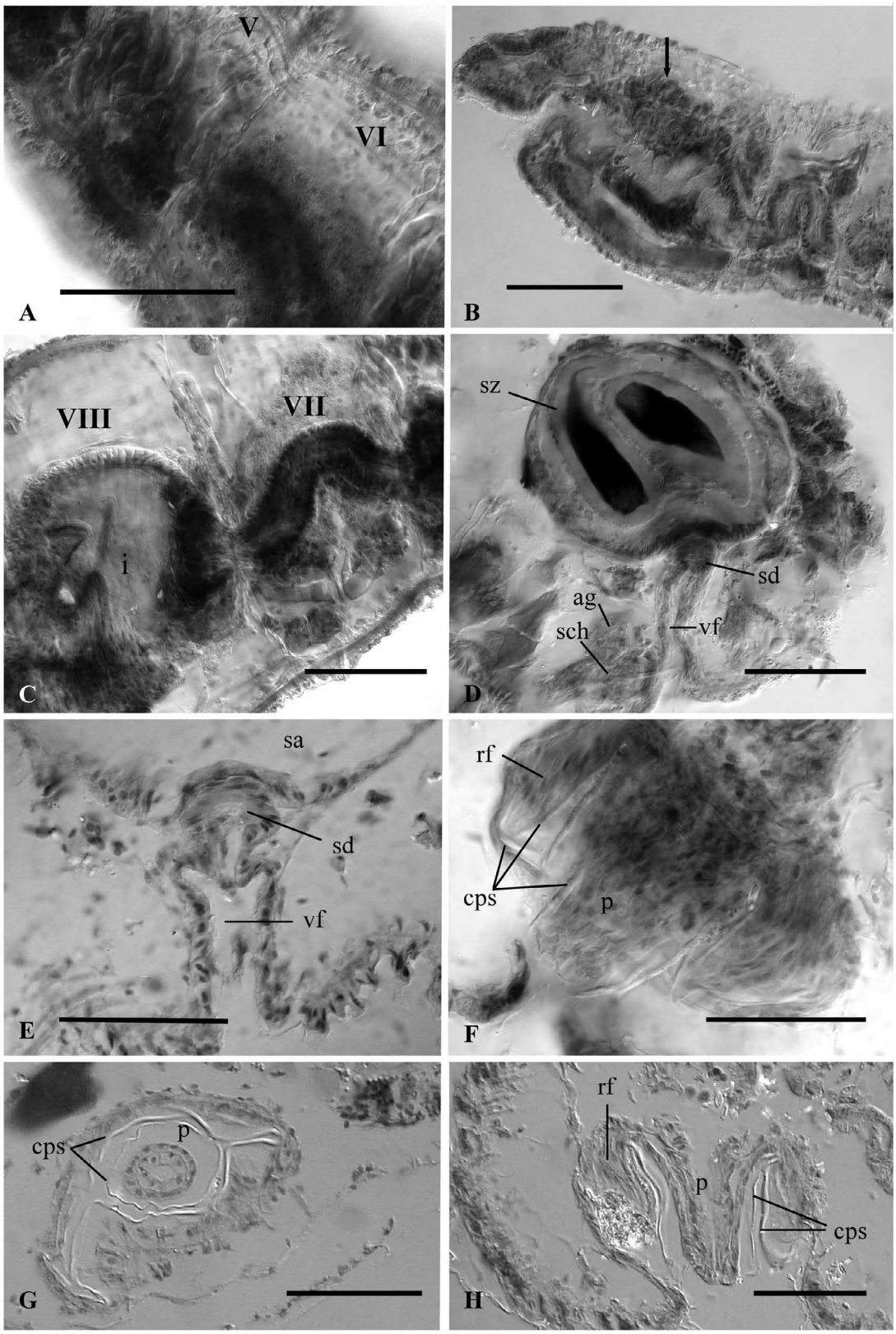

FIGURE 4. *Isochaetides gianii* **n. sp.** A: commissural blood vessels in body segments V and VI; B: anterior end of the body, arrow pointing to the glands on the pharynx, in segment III; C: sudden transition to the intestine, from segments VII to VIII; D: spermatheca; E: detail of spermatheca in a histological section; F: penial sac with penis; G: cross histological section of penial sac; H: longitudinal histological section of penial sac. **Abbreviations**: ag: accessory gland, cps: cuticular penial sheath, i: intestine, p: penis, rf: ring fold of the penial sac, sa: spermathecal ampulla, sch: spermathecal chaeta, sd: spermathecal duct, sz: spermatozeugmata, vf: ventral fold of the spermatheca. **Scale bars**: A–D 100 µm, E–H: 50 µm.

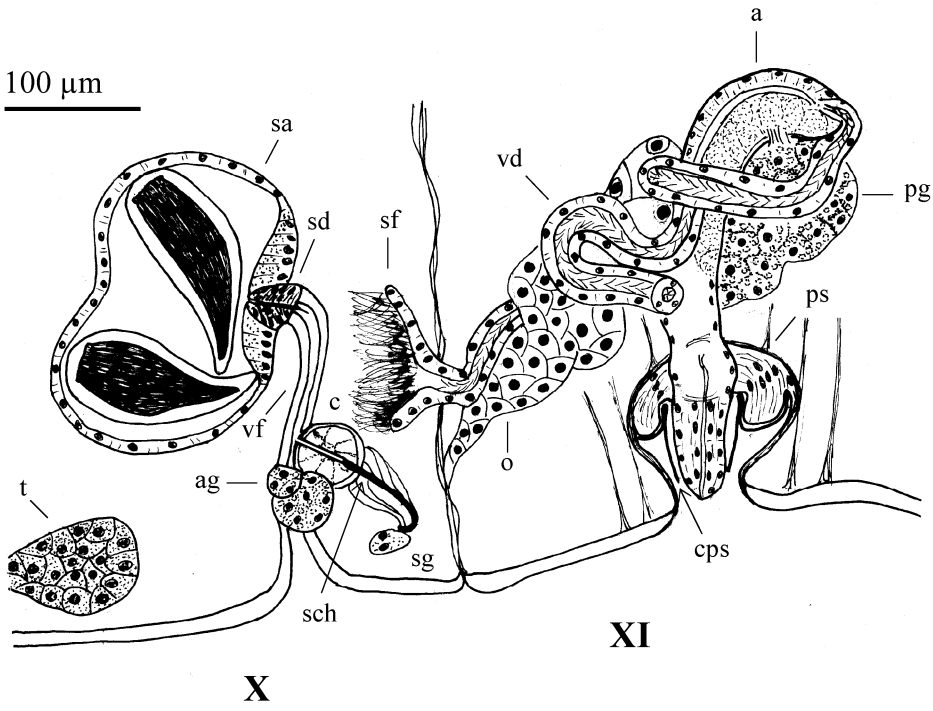

FIGURE 5. *Isochaetides gianii* **n. sp.** General diagram of the reproductive organs. X and XI: segment number. **Abbreviations:** a: atrium, ag: accessory gland, cps: cuticular penial sheath, o: ovary, pg: prostate gland, ps: penial sac, sa: spermathecal ampulla, sch: spermathecal chaeta, sd: spermathecal duct, sg: chaetal gland, t: testis, vd: vas deferens, vf: ventral fold of the body wall.

and ciliated throughout, a penis within a penial sac, and modified spermathecal chaetae. Other close genera with similar characteristics of the male duct are *Tasserkidrilus*, *Arctodrilus*, *Troglodrilus* and *Haber*, the first three lacking modified genital setae, and the latter with both spermathecal and penial chaetae modified. The new species has modified spermathecal setae in X and no penial chaetae, as well as a general structure of the male duct which can be present in either *Isochaetides* or *Lamadrilus*. These include the vas deferens joining the atrium subapically, and the expanded proximal end of the atrium, which has two layers of glandular cells: a dark, densely granulated layer in the concave side and a light layer in the convex side. The new species is well distinguished from other species of *Isochaetides* or *Lamadrilus* by a combination of characters that include: hair chaetae and pectinates in dorsal bundles, grooved spermathecal chaetae associated with chaetophores and glands, a well-developed penial sac lined by a cuticle layer with a proximal ring fold, and a cuticular penial sheath (not asymmetrical or in the form of a spade).

Due to the feeble differences between the genera *Isochaetides* and *Lamadrilus*, the new species was first identified as *Lamadrilus* sp. (Achurra & Rodriguez 2008), mainly based on the presence of hair chaetae. Most species in this genus have only bifid somatic chaetae, except *L. bazikalovae* (Chekanovskaya, 1975), which presents different rates of reduction in hair chaetae and pectinates (Timm 1998). However, we agree with Brinkhurst and Kathman (1983) that the absence of species with hair chaetae in a genus should not be a problem for the inclusion of a new species with hair chaetae; and *I. gianii* **n. sp.** has been classified on the basis of the structure of the reproductive organs. The genus *Isochaetides* Hrabě, 1966 includes species that possess modified spermathecal chaetae (except *I. distinctus* Semernoy, 2001). These are characteristically grooved in the distal end (except for *I. michaelseni* Lastočkin, 1937, which has bifid genital chaetae), and generally appear associated with spermathecal glands. In several species, these glands are poorly described, but their remarkable distal position with respect to the spermathecal chaeta, and their structure (sometimes only seen in drawings), is similar to the chaetophores that we have described in *I. gianii* **n. sp.** Chaetophores

associated with spermathecal chaetae are also known in other oligochaete genera [e.g. the rhyacodriline *Protuberodrilus tourenqui* Giani & Martínez-Ansemil, 1979 and the telmatodriline *Alexandrovia ringulata* (Sokolskaya, 1961)]. On the other hand, only one of the six known species of *Lamadrilus* (*L. sorosi* Timm, 1998) has modified spermathecal chaetae of the same type as that described in *Isochaetides* species, as well as a chaetophore and accessory glands associated to the chaetal sac.

The two different epithelial layers of the atrium described in the new species are probably equivalent to the dark and light layers described in *L. sorosi* by Timm (1998), and are also reminiscent of the layers shown in drawings of some *Isochaetides* species. This histological characteristic has been confirmed in the examination of type specimens of *I. freyi* (Brinkhurst, 1965) (Fig. 6A), although it has probably been overlooked in most descriptions, not only in species of these genera but also in other tubificines.

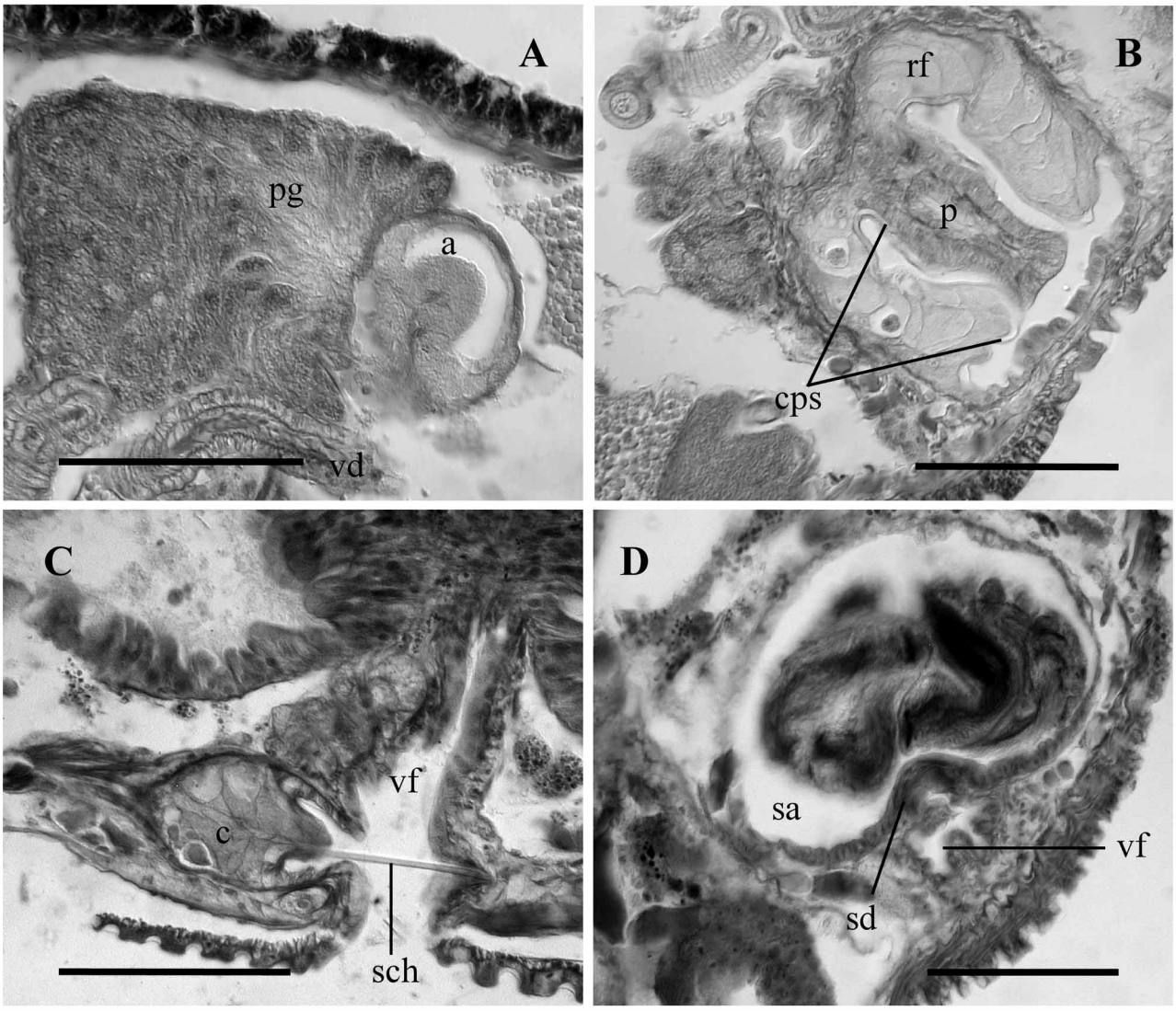

FIGURE 6. *Isochaetides freyi* (Brinkhurst, 1965). A: histological section of the atrium; B: penial sac with penis lined by cuticle; C: ventral fold of the body wall, where the spermathecal duct opens; D: spermatheca. **Abbreviation**s: a: atrium, c: chaetophore, cps: cuticular penial sheath, p: penis, pg: prostate gland, sa: spermathecal ampulla, sch: spermathecal chaeta, sd: spermathecal duct, vd: vas deferens, vf: ventral fold of the body wall. **Scale bars**: 100 µm.

The presence of a penial sac with an apical ring fold is known in other *Isochaetides* species (e.g. *I. freyi*) (Fig. 6B). Several lateral folds are present in the penial sac of some *Lamadrilus* species (e.g. *L. sorosi*), but never in the form of an ental ring fold. The presence of a short, mostly asymmetrical cuticular penial sheath

(sometimes with a spade-shaped distal appendage) is a diagnostic character in *Lamadrilus*; however, the genus incorporates different types of penial sheaths (sometimes variable in shape within the same species, with a more or less marked asymmetry, see Timm, 1998: Figs. 45–50). The diagnosis of the genus *Isochaetides* by Snimschikova (1998) describes the cuticular penial sheath as thin or absent and Semernoy (2004) describes it as more or less thin, either of uniform width or conical but without oblique cut on its distal end. In fact, all *Isochaetides* species known to date, except for *I. lacustris*, have a thin cuticular penial sheath or a thin cuticular covering on the penis (although thick in *I. distinctus* Semernoy, 2004). The examination of the type specimens of *I. freyi* have revealed a conspicuous cuticular layer, covering the inner side of the penial sac, the large ental fold and the penis, and taking the shape of their contour (Fig. 6B), in a similar way to that in the new species.

The structure of the spermatheca is not commonly used as a diagnostic character in tubificines. However, in *Isochaetides* the short spermathecal ducts, associated with modified spermathecal chaetae located in adjacent glandular sacs (referred to as chaetophores in present work), appear to be a very common feature. The structure of the spermathecal duct is contentious and what is described here as a short spermathecal duct opening at the ental end of a ventral body fold, appears to be interpreted as a short ental sphincter of the spermathecal duct by Semernoy (2004) in *I. peniacerus* Semernoy, 2004, *I. compactus* Semernoy, 2004 and *I. septatus* Semernoy, 2004. The histology of the duct in the new species reveals that the cells are not muscular, and the irregular aperture at the body surface does not have the structure of a pore, typically formed by cells arranged in a circle around the opening. Short spermathecal ducts, opening into ventral folds of the body wall, associated with grooved spermathecal chaetae and chaetophores, are also present in *I. freyi* (Fig. 6C, D). These characters are shared by most *Isochaetides* species, and we have considered them together with the general structure of the male duct and penis, for the classification of the new species as a member of the genus *Isochaetides*.

The finding of an *Isochaetides* species in karstic areas in northern Iberian Peninsula is noteworthy, since the genus is represented mainly in eastern Europe (Timm & Veldhuijzen van Zanten 2002) and Asia (many species in Lake Baikal, see Semernoy 2004), although three species have been described in America: *I. freyi* (Brinkhurst, 1965) and *I. curvisetosus* (Brinkhurst & Cook, 1966) [considered as a probably synonym of *I. hamatus* (Moore, 1905) after Brinkhurst, 1986] in North America; and *I. lacustris* (Černosvitov, 1939) in South America.

***Lophochaeta ignota* Štolc, 1886**

(Figures 7–9)

Lophochaeta ignota Štolc, 1886
Lophochaeta ignota Štolc: Štolc 1888
Tubifex filum Michaelsen, 1901
Tubifex longiseta Bretscher, 1905
Tubifex ignotus (Štolc): Laakso 1969. Hrabě 1981. Chekanovskaya 1981
For references before 1971 see Brinkhurst and Jamieson 1971

Examined material. From Apraiz karstic spring (6 September 2005), UTM coordinates X:524225, Y:4801350, Z:22. One whole-mounted in lactophenol, 3 sectioned, 4 dissected (one of them not fully mature) and 24 specimens preserved in alcohol 70% (at different stages of maturity). From Iturgoien cave (6 September 2005) one dissected specimen not fully mature.

Localities. Apraiz spring and Iturgoien cave (X:524217, Y:4801296, Z:21) both are very close and connected subterranean sites in Busturia (Santa Eufemia–Ereñozar karst unit, Biscay, Spain) (6 September 2005).

Description. Long worms, body length of preserved mature specimens up to 40 mm with 160 segments; width in segment VIII 0.3–0.4 mm; and down to 0.15 mm in the posterior region of the body.

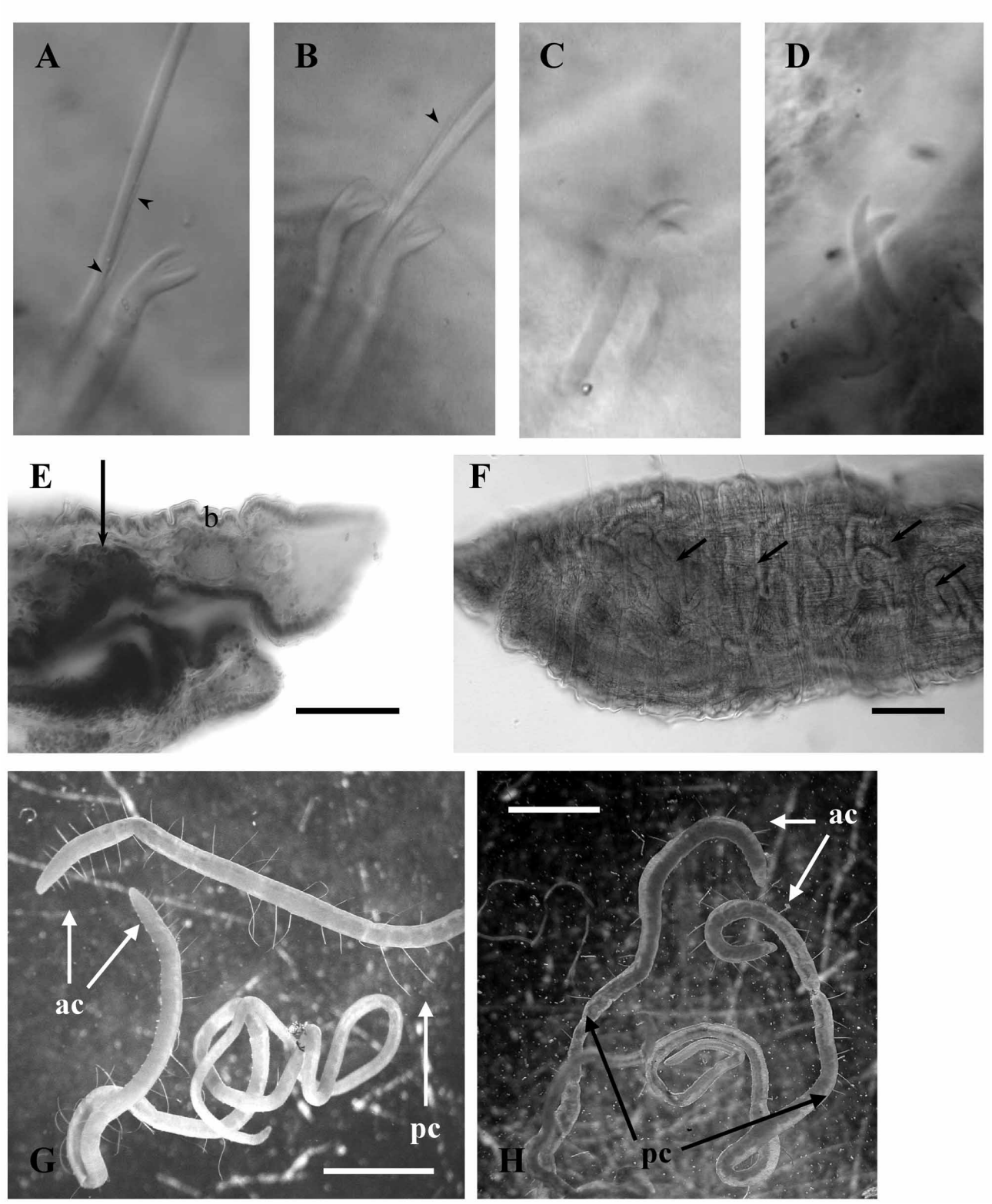

FIGURE 7. *Lophochaeta ignota* Štolc, 1986. A and B: dorsal pectinate and barbed hair chaetae in preclitellar segments (arrows pointing to barbs); C: anterior ventral chaeta; D: posterior ventral chaeta; E: anterior part of the body, arrow pointing to the glands on the pharynx, in segment III; F: commissural blood vessels in several consecutive segments of the anterior part of the body (see arrow heads); G and H: general view of specimens from Estonian (G) and Russian (H) populations, arrows pointing to anterior and posterior hair chaetae. **Scale bars**: E–F: 100 µm, G and H: 2 mm.

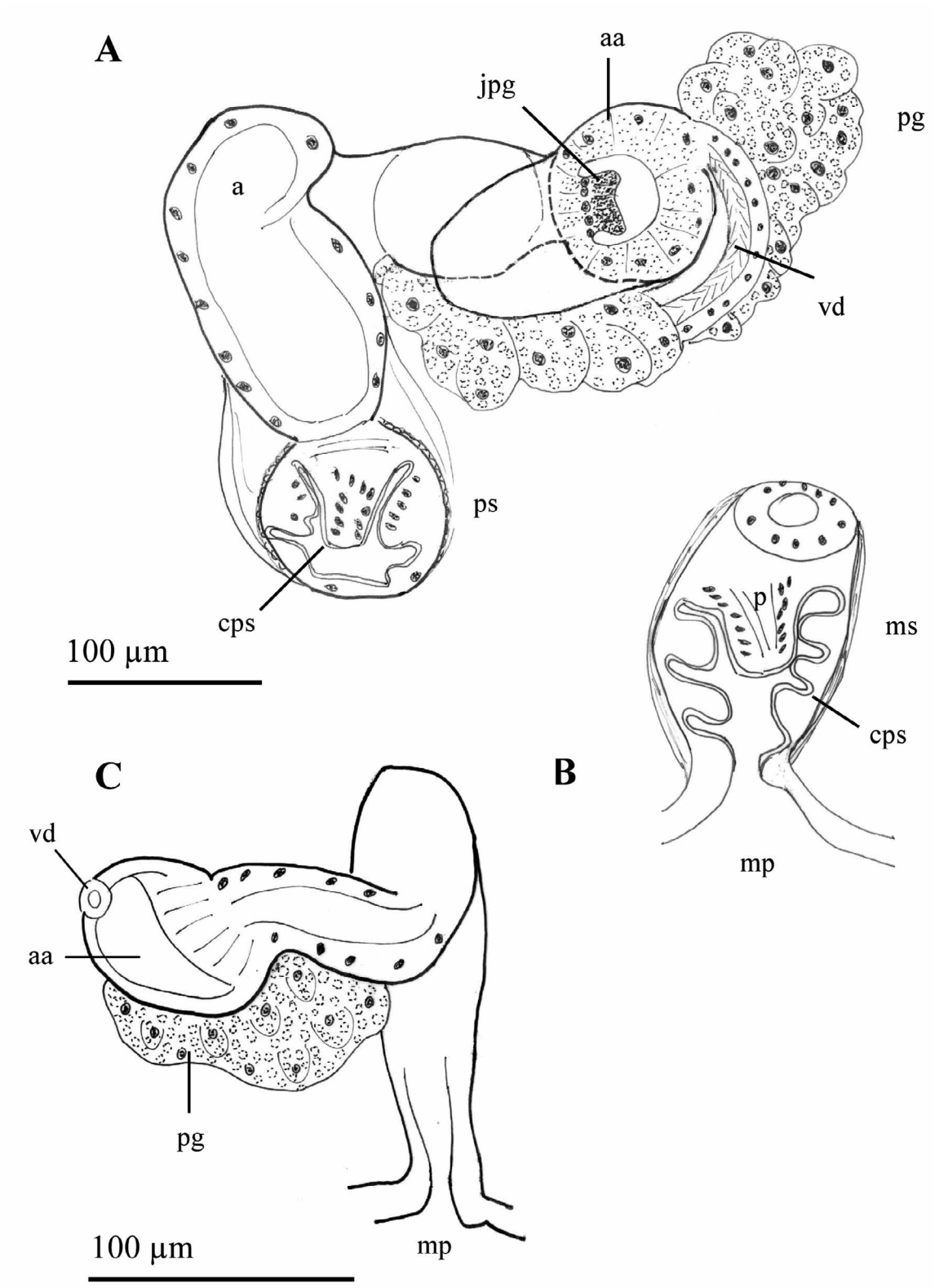

FIGURE 8. *Lophochaeta ignota* Štolc, 1886. A: general diagram of the male duct. X and XI: segment number; B: detail of the penial sac; C: male duct of incompletely mature specimen. **Abbreviations:** a: atrium, aa: atrial ampulla, cps: cuticular penial sheath, jpg: junction of the prostate gland, mp: male pore, ms: muscular strands, p: penis, pg: prostate gland, ps: penial sac, vd: vas deferens.

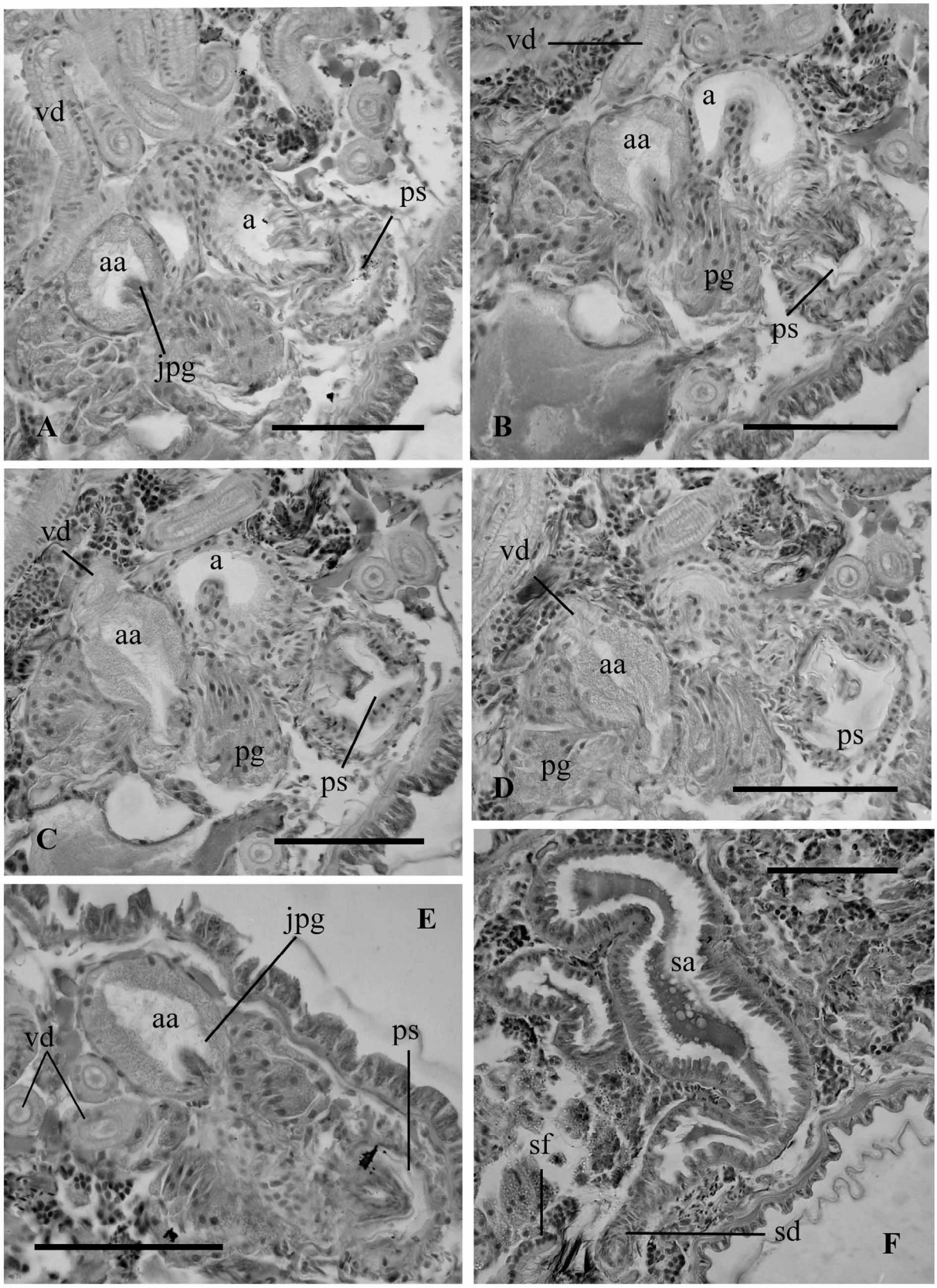

FIGURE 9. *Lophochaeta ignota* Štolc, 1886. A to D: consecutive histological sections of the male duct, showing opposite junctions of prostate gland and vas deferens to the atrial ampulla; E: other histological section of the male duct; F: spermatheca. **Abbreviations:** a: atrium, aa: atrial ampulla, cps: cuticular penial sheath, jpg: junction of the prostate gland, pg: prostate gland, ps: penial sac, sa: spermathecal ampulla, sd: spermathecal duct, sf: sperm funnel, vd: vas deferens. **Scale bars**: 100 µm.

Prostomium rounded, 120–135 µm long and 115–145 µm wide. Body wall consisting of a muscular layer 5–7 µm thick, and an epidermal layer 5–10 µm thick covered by a cuticle of 2–3 µm. Anterior region of the body with secondary annulation; in two examined mature individuals it was apparent from segment II to V and II to VIII, respectively. Clitellum in segments XI–XII, not much elevated. One pair of spermathecal pores in X, close and lateral to the ventral bundles of chaetae. When completely mature (with eggs into the egg sac), one pair of male pores open close to ventral bundles of chaetae, in a hollow formed in the ventral side of segment XI.

Dorsal bundles of chaetae consist of hair and pectinate chaetae. Hair chaetae very long and hispid (Fig. 7A, B) covered by fine bristles visible under 400x magnification. In preclitellar segments 1–2 hairs per bundle, one in postclitellar segments and absent at the hind part of the body. Length of hair chaetae grows backwards, up to 350 µm long in segment III; up to 480 µm in V; up to 650 µm in VIII (approx. 2 times the body diameter) and up to 850 µm (2 to 3 times the body diameter) in postclitellar segments. In preclitellar dorsal bundles, 2–3 characteristic pectinate chaetae per bundle, 88–95 µm long, with distal nodulus, the proximal part of the chaeta straight and the distal part curved; stout teeth up to 7 µm long, with 2 intermediate thin teeth of about equal length (Fig. 7A, B)). In posterior segments, one pectinate chaeta per bundle, 60–78 µm long, with shorter teeth and gradually losing the characteristic curved shape of the distal part of the chaeta towards the hind region of the body, where regular sigmoid chaetae without intermediary teeth are found. Ventral bundles in anterior segments with 3–4 bifid chaetae (52–82 µm long), distal tooth longer than proximal one (Fig. 7C); in postclitellar segments 2–3 bifid chaetae, 68–73 µm long, with distal tooth shorter and thinner than proximal one (Fig. 7D). No modified genital chaetae.

Brain back to intersegment 1/2. Coelomocytes not visible within the coelomic cavity in most specimens, although in some occasions small ones (*ca* 3 µm diameter) were observed. Typical muscular dorsal pharyngeal pad not developed. Pharyngeal glands from segment III back to segment VI, dorsal to the pharynx in segment III (Fig. 7E), lateral and ventral to the gut in IV and V, and ventral in VI. A layer of chloragogen cells on the digestive tract, from segment VI, backwards. Oesophagus opens gradually into the intestine. Dorsal and ventral vessels very apparent throughout the body and commisural vessels noticeable in anterior segments (Fig. 7F).

One pair of testes in X and one pair of ovaries in XI. Sperm sacs developed anteriorly to segment IX and back to segment XIII. Egg sac can reach segment XV. All following measurements on the male duct were obtained from egg-bearing individuals. Male funnel 100–107 µm high, attached to the septum in 10/11. Very long vas deferens, forming numerous loops in segments XI and XII, ciliated and about the same diameter throughout its length, varying in different specimens from 30 to 40 µm (in one specimen briefly narrowing just before the junction with the sperm funnel); epithelial layer 7.5–10 µm thick. Atria tubular in segment XI, approximately 275–325 µm long, 34–43 µm diameter, and covered by a very thin muscular layer. Maximum diameter of atrium up to 60 µm in the distal portion; atrium folds several times, and enters the penial sac through a narrow section *(ca.* 30 µm diameter). Atrium expands entally, forming an oval ampulla (70–80 µm wide, and 105–150 µm long) (Fig. 8A). Atrial epithelium simple in the ectal section, and glandular (finely granular) in the ental ampulla. Epithelial cells at the atrial ampulla higher (up to 25 µm) than those in the other sections of the atrium (13–18 µm). Vas deferens enters the distal part of the atrium opposite to the junction of the compact prostate gland (Fig. 9A–D). Atrium opens into elongated penial sac, 100 µm wide, reinforced by both longitudinal and circular musculature. In fully mature specimens, the internal walls of the penial sacs are covered by a thin cuticle, and have several lateral folds (Fig. 8B and 9B–E). Short, conical penis (up to 37 µm long and 37 µm maximum diameter) is lined with a cuticular layer thinner than that of the body wall; its outline follows the shape of the penis. The penis is not free within this cuticular sheath. Female funnels were not observed.

One pair of spermathecae in segment X. Spermathecal ampulla elongated, up to 425 µm long and 132 µm diameter in the examined specimens, gradually narrowing towards the spermathecal duct, which is relatively short (50–70 µm long and 25 µm wide) (Fig. 9F). Spermathecal epithelium up to 10 µm high. Undetermined

matter fills the lumen of the ampulla, with a smooth uniform surface that resembles a liquid, and no remnants of spermatozeugmata or spermatozoids were observed.

Distribution and habitat. The species has been widely reported in lakes and rivers of the Holarctic region, Africa (Tunisia and South Africa) and South America (Lake Titicaca). In the Iberian Peninsula, it has been reported in epigean waters, usually immature; in karstic springs of the study area it has been found mature for the first time.

Taxonomic remarks. The description of the new material of the species agrees with the diagnosis of *Lophochaeta ignota* by Štolc (1886, 1888), whose description was meticulous on nerve and vascular systems but poor on the reproductive organs. Štolc did not measure the length of the hair chaetae but mentioned their hispid nature. The new material also agrees with the diagnosis of *Lophochaeta* by Holmquist (1985), being remarkable the presence of hispid (barbed) hair chaetae; the tubular shape of the atrium, with an ental ampulla, where prostate and vas deferens enter opposite each other; and the long and throughout ciliated vas deferens. The position of spermathecal pores, lateral in *Tubifex* and ventral in *Lophochaeta*, could be a character subject to variation at the species level, although Holmquist introduced it in the diagnosis of the genera *Tubifex* (as lateral) and *Lophochaeta* (as ventral). This variability can also be applied for the shape of spermathecal ampulla (rounded *vs.* sac-like) or for the length of the spemathecal duct (short *vs.* long).

The synonymy of *Tubifex filum* with *Lophochaeta ignota* (proposed by Hrabě 1962, and widely accepted) is admitted by the authors, although we recognize that there are some blank gaps in the original description of the species. Thus, Štolc (1886, 1888) did not mention the presence of exceptionally long hair chaetae in the diagnosis of *Lophochaeta ignota* although this character was stated in subsequent descriptions of the species (Michaelsen 1901, Hrabě 1962). By other side, *Tubifex filum* was described by Michaelsen (1901) as a long, thin and threadlike worm, with 2 (3) very fine and long hair chaetae with maximum length behind the clitellum (0.9 mm), but there was no mention to barbs. According to our measurements in North European populations of *L. ignota*, the length of the hair chaetae can be variable (up to 550 µm in postclitellar segments, in the Russian population; up to 1350 µm in postclitellar segments, in the Estonian population) (Fig. 7G, H). Regarding to the barbs on the hair chaetae, all examined specimens from different geographic areas have hispid chaetae, which were observed at a magnification of 400x and with interferential contrast.

The general description of the male duct of the species *L. ignota* by Hrabě (1962, 1981, as *Tubifex ignotus*) mostly agrees with ours, except that he states that the vas deferens gradually dilates from the ental (22 µm diameter) to the ectal part (32–40 µm diameter). He also described the junction of vas deferens with the atrium as apical, in the small spherical ampulla of the atrium. It is interesting to note that this general sketch of the atrium is similar to that found in individuals which have not formed eggs yet (see Fig. 8C). Our examined specimens have glandular epithelium mostly restricted to the apical ampulla while atrial walls were described as "glandular for the most part" by Chekanovskaya (1981: 340). She also stated that atrium "posteriorly narrows abruptly to 44 µm", probably referring to the narrowing that occurs just before entering the penial sac.

Hrabě (1981) described the penis of *L. ignota* (as *T. ignotus*) being cylindrical and covered by a strong cuticle, but not forming a cuticular tube of permanent shape. In our specimens, the cuticle although well visible, does not appear strong and it conforms to the shape of the penis, which is not free inside a cuticular sheath. This can be interpreted in the same way as Hrabě's description. Chekanovskaya (1981) described the penis of the species as "small and rounded" within a folded penial sac. In the examined specimens from our collection, the penis is conical and short in relation to the length of the penial sac, which is also folded (Fig. 8B).

The general shape and structure of the spermatheca in present description agrees with the description given by Štolc (1886, 1888), Hrabě (1962, 1981) and Chekanovskaya (1981). Spermatozeugmata were only mentioned by Hrabě (1962) but drawings or detailed descriptions were lacking. The examination of specimens from Russia revealed the presence of sperm into the spermathecal ampulla, but without forming spermatozeugmata (observation confirmed by M. Ferraguti).

Discussion

Research on the groundwater oligochaete fauna in karstic areas of the Iberian Peninsula has demonstrated significant diversity: 21 stygobiont species (including the two new species described in the present paper), of which 11 are endemic to one karst unit (Giani *et al.* 2001; Camacho *et al.* 2006; Achurra & Rodriguez 2008).

In recent years, the knowledge of the genus *Gianius* has slowly grown (11 species known so far, including *G. navarroi*), and the presence of this genus in freshwaters has been confirmed in different parts of the world, both in groundwaters (Southern Europe: Giani *et al.* 2001; Juget & des Chatelliers 2001) and in riverine habitats, probably hyporheic or connected with groundwaters (Turkey: Arslan *et al.* 2007; North America: Farara & Erséus 1991). In 1992, Erséus reviewed the subfamily Phallodrilinae and erected the genus *Gianius*, which grouped most species known to date in the subterranean freshwater habitats. Unfortunately the species are usually found at very low density, but this could be due to deficiencies in the sampling method, or even to the possibility that the main habitat of the subfamily in freshwater (superficial waters or groundwaters) has not been adequately sampled yet.

The diagnosis of the genus *Isochaetides* Hrabě, 1966 was emended by Brinkhurst (1981), next by Snimschikova (1998) and more recently by Semernoy (2004). *Isochaetides* can be distinguished from *Lamadrilus* Timm, 1998 by the thin, about symmetrical cuticular penial sheath, which is not much thicker than the normal cuticular layer of the body wall (absent in *I. lacustris*). In *Lamadrilus*, the cuticular penial sheath is mostly asymmetrical, and does not conform to the shape of the penis, whereas in *Isochaetides* it does. In addition, the penial sac is cuticle-lined in some *Isochaetides* species [e.g. *I. grubei* (Michaelsen, 1905) and *I. frey*i], similar to *I. gianii* **n. sp.**, which also has a symmetrical, cuticular penial sheath.

Holmquist (1985) reviewed the genus *Tubifex* Lamarck and other related genera, reinstated the previous name *Lophochaeta* for the species *L. ignota* Štolc, 1886 and described a new species *L. paucipilifer* Holmquist, 1985, collected in lakes of Alaska. We accept her proposal of distinguishing the genus *Tubifex* from *Lophochaeta* based on differences in several taxonomic characters related to the male duct structure. Following her diagnosis, the genus *Tubifex* is characterised by the presence of a bipartite vas deferens (a proximal ciliated section and a distal non-ciliated one), entering the atrium apically or subapically (i.e. close to the prostate gland junction, in the concave part of the bent atrium), and the penis is lined by a cuticle within a penial sac having an ental circular fold associated with strong circular muscle bundles. The genus *Lophochaeta* is defined by Holmquist (1985) by a number of characters which readily separate it from *Tubifex*, such as a long and thick vas deferens that is ciliated throughout (not bipartite), entering the atrium subapically, opposite the prostate gland junction, long tubular atrium with proximal end distended, and a penis lined with a cuticle within a penial sac devoid of an ental circular fold.

Lophochaeta paucipilifer has been considered *species inquirenda* by Brinkhurst (1986). However, on the basis of the original diagnosis this species can be well distinguished from *L. ignota* by a long penis (compared to the size of the penial sac). The atrium is proximally distended in both species, forming an ental ampulla, but in *L. paucipilifer* it has a constant diameter in the remaining section, whereas in *L. ignota* it has several folds and narrows just before entering the penial sac. The atrial epithelium in *L. paucipilifer* is glandular throughout, with "slight" histological differences between distal and proximal parts (Holmquist 1985: pg. 319), while in our specimens of *L. ignota* glandular cells are mostly restricted to the proximal region. Chekanovskaya (1981) described the atrial epithelium in *L. ignota* as glandular throughout, although minor differences in the histology could be subject to physiological differences in the individuals related to the mating process. Finally, the shape of spermatheca clearly distinguishes *L. paucipilifer* from *L. ignota*, the former with a round, sac-like ampulla well separated from the duct, while in the latter the duct is nearly indistinctly demarcated from the sac-like ampulla. Thus, *L. paucipilifer* appears to be a species well separated from *L. ignota* based not only in somatic characters (pectinates not curved like in *L. ignota*, and hair chaetae only in the first few segments with barbs "hard to detect") but also in several features of the male duct. Future descriptions of the male duct of *L. ignota* are necessary to clearly ascertain the identity of the many records in

the world, since most of them are exclusively based in the general outline of the body (thin and long) and the shape of the chaetae, characters that may obscure several additional species in different parts of the world.

Acknowledgements

We are indebted to Edelweiss and ADES speleological groups for helping in cave prospections. We also express our gratitude to Dr. Ana I. Camacho for providing material from Ojo Guareña cave, to Dr. Tarmo Timm for his valuable taxonomic comments and for making available the Russian and Estonian specimens of *Lophochaeta ignota* and the type specimens of *Lamadrilus sorosi*, and to the National Museum of Natural History (Smithsonian Institution) of Washington (USNM) for the loan of *Isochaetides freyi*. We also wish to thank Steve Fend for kindly revising our English text and for his especially helpful suggestions and Christer Erséus for his valuable comments on the manuscript. The junior author has got a predoctoral financial support by the UPV/EHU (2007-2011). The Basque Government Department of Regional Planning and Environment partially supported the investigation in the karst of Santa Eufemia-Ereñozar.

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