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Two new species of *Fridericia* (Annelida: Enchytraeidae) from Hungarian caves

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[3] urn:lsid:zoobank.org:author:F0CD45C8-0140-4E52-A996-080FE186EB72

Abstract. Cave research in Hungary has developed a lot in the last decade. As a part of this progress, enchytraeid specimens were collected from Hungarian caves and were subsequently characterized by comparative morphological and molecular taxonomic analyses. Molecular phylogenetic studies based on ITS, CO1 and H3 sequences and morphological results confirmed that these specimens represented two species new to science. The descriptions of *Fridericia baradlana* sp. nov. and *Fridericia spelaeophila* sp. nov. are presented in this paper.

Keywords. Clitellata, troglophile, morphological and molecular taxonomy, Northern Mountain Range of Hungary.

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Introduction

Aquatic oligochaetes living in the subterranean environment have been studied extensively and significant results have been published related to several aspects of their biology and ecology (see the review of Creuzé des Châtelliers *et al*. 2009). Contrary to this, only few data are available on cave enchytraeids, and in most of these publications Hungarian cave habitats were characterized. Outside Hungary, only Botea (1973, 1984) studied such environments, and he described seven new species from Romanian caves, but these novel species later proved to be *nomen dubia* (Schmelz 2003; Schmelz & Collado 2010). Unfortunately, his material was lost, therefore uncertainties exist regarding the published data.

In Hungary, Professor Endre Dudich played a prominent role in the study of cave animals; in his monograph published in 1932, he recorded 262 species from the Baradla cave (part of the Baradla-Domica cave system, Aggtelek Karst, Hungary) (Dudich 1932). In further research carried out under his supervision (Dózsa-Farkas 1970, Dózsa-Farkas 1974), this was succeeded by the detection of 13 enchytraeid species and additionally by the description of four species new to science (*Cernosvitoviella aggtelekiensis* Dózsa-Farkas, 1970*, Enchytronia christenseni* Dózsa-Farkas, 1970*, Fridericia reducata* Dózsa-Farkas, 1974*,* and *F. semisetosa* Dózsa-Farkas, 1970). Subsequent studies have confirmed that these species (including the newly described ones) cannot be considered troglobionts, since they were later found in various aboveground habitats (Dózsa-Farkas 2019).

Cave research in Hungary has developed considerably in the last decade (e.g. Erőss *et al*. 2012; Anda *et al*. 2017). From the Baradla-Domica cave system, 583 invertebrate taxa have already been reported (Salamon *et al*. 2015). Recently, zoologist caver colleagues also collected enchytraeid specimens, namely from the caves Baradla, Szepesi and Láner Olivér and from the Kis-kőháti shaft (these are located in the Northern Mountain Range of Hungary, the latter three in the Bükk Mts). Based on the results of traditional morphological and molecular taxonomic analyses, these specimens are considered to represent two species new to science. In this article, the descriptions of these two new enchytraeid species are given.

Materials and methods

Abbreviations

CO1 = Cytochrome c Oxidase subunit I
ELTE = Eötvös Loránd Tudományegyetem (Eötvös Loránd University)
H3 = Histone 3
ITS = Internal Transcribed Spacer
PCR = Polymerase Chain Reaction

Methods of morphological examination

In 2013 and 2014, specimens were picked up individually and fixed immediately in 96% ethanol. In 2015 and 2018, live worm specimens were brought into the laboratory together with some pieces of decaying wood. From the decaying wood and from the clay on it, several worms were extracted by the wet funnel method (O'Connor 1962). The extracted enchytraeids were observed and measured *in vivo*, then fixed and preserved in 70% ethanol. The majority of these specimens and the majority of those fixed on site (collected in 2013 and 2014) were stained with borax-carmine, and then passed through an ethanol dehydration series (from 70% to absolute), mounted temporarily in clove oil, then permanently in Euparal between two coverslips. Hence the worms were observable from both sides (Schmelz 2003). Some specimens were prepared without staining. All the important morphological characters were recorded, drawn and photographed (Axio Imager.A2 microscope with differential interference contrast illumination, AxioCam MRc 5 [Zeiss] digital camera, Axiovision software) *in vivo* and fixed alike. Due to their large body size, fixed specimens of *F. spelaeophila* sp. nov. were often cut into two parts and the forepart was opened dorsally and stained. Slides containing the forepart of the whole-mounted worms were marked with 'a' and slides with the posterior parts with 'b'. In all micrographs presented in this study, the orientation of specimens is the same; the head is either on the left side or at the top of the picture.

Selected material was catalogued with letters for the holotypes ('F') and paratypes ('P'), with collection numbers and with slide numbers, and was deposited in the collection of the Department of Systematic Zoology and Ecology, ELTE.

Methods of molecular analysis

Genomic DNA was extracted from the body end or from the entire individual preserved in absolute ethanol using the DNeasy Blood & Tissue Kit (Qiagen) according to the instructions given by the manufacturer. The mitochondrial cytochrome c oxidase subunit I (CO1) gene, the nuclear histone 3 (H3) gene and the nuclear ribosomal ITS region (ITS) were amplified separately by polymerase chain reaction (PCR) using the primer pairs HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') (Folmer *et al.* 1994), H3a-F (5'-ATG GCT CGT ACC AAG CAG ACV GC-3') and H3a-R (5'-ATA TCC TTR GGC ATR ATR GTG AC-3') (Colgan *et al*. 1998), ETTS1 (5'-TGC TTA AGT TCA GCG GGT-3') and ETTS2 (5'-TAA CAA GGT TTC CGT AGG TGA A-3') (Kane & Rollinson 1994), respectively. If amplification failed in the case of the H3 gene, one additional primer set (designed by AllGenetics, A Coruña, and used here with permission of ECT Oekotoxikologie GmbH, Flörsheim) was applied, H3Frid-M13F (5'-GTA AAA CGA CGG CCA GTT ACC AAG CAG ACG GCH CGY-3') and H3Frid-M13tR (5'- GCG GAT AAC AAT TTC ACA CAG GGG CGT GAA TBG CRC ACA GGT-3'). PCRs were performed as described in detail previously by Dózsa-Farkas *et al*. (2015). Sanger sequencing was carried out by the LGC Genomics GmbH (Berlin, Germany) or by the Biomi Ltd. (Gödöllő, Hungary), while phylogenetic analyses including the search for the best fit model were performed with the MEGA 7 software (Kumar *et al*. 2016). The following nucleotide substitution models were applied in the analyses: for the ITS, K2+G+I; for the CO1, TN93+G+I; and for the H3, K2+G. Concatenation was carried out with Sequence Matrix version 1.8. (Vaidya *et al*. 2011). Bayesian analysis was performed with the Markov Chain Monte Carlo algorithm in two simultaneous, completely independent analyses running for 1 000 000 generations using MrBayes version 3.1 (Huelsenbeck & Ronquist 2001). Posterior probabilities were calculated after the two independent runs had reached convergence, and the first 25% of the calculated trees were discarded. The concatenated phylogenetic tree comprises those specimens, which had all of the three studied regions sequenced. The obtained sequences were deposited in GenBank under the following accession codes: MK560154–MK560158 (ITS), MK580963–MK580966 (CO1) and MK562739–MK562744 (H3). Additional sequences determined in previous studies used for comparison are given in Table 1.

Results

Species descriptions

Class Clitellata Michaelsen, 1919
Order Enchytraeida Kasprzak, 1984
Family Enchytraeidae Vejdovský, 1879
Genus *Fridericia* Michaelsen, 1889

Fridericia baradlana sp. nov.
urn:lsid:zoobank.org:act:692C37DF-0429-44CA-B989-495C256D2D43
Figs 1–3

Diagnosis

The new species can be recognized by the following combination of characters: (1) medium sized (9–15 mm *in vivo*); segments 42–61; (2) chaetae maximum 4–5 per bundle; (3) clitellum weakly developed, girdle shaped; (4) body wall 25–35 μm thick, cuticle thin (<1 μm); (5) oesophageal appendages long with many branches at the end; (6) all pharyngeal glands united dorsally; (7) five pairs of preclitellar nephridia; (8) coelomo-mucocytes scarce, a/c-type, lenticytes numerous and large; (9) dorsal vessel from XIX–XXI; (10) chylus cells in XII–XIV, occupying 2 segments; (11) bursal slit T-shaped; (12) seminal vesicle in XI, not brown; (13) subneural glands in XIII–XIV; (14) sperm funnels approximately

as long as half the body diameter, collar as wide as funnel body, spermatozoa 250–320 μm long, heads 110–150 μm *in vivo*; (15) spermatheca with two long, arm-like diverticula variously bent or bifurcate in proximal part; ectal ducts of variable length with two ectal glands and ampullae fused proximally with common opening into oesophagus.

Etymology

Named after the type locality, Baradla cave.

Material examined

Holotype

HUNGARY • Baradla cave (Aggtelek Karst), entrance of Styx branch, from bits of decaying wood (these are remains of an old wooden bridge); 48°28′55.0″ N, 20°30′0.85″ E; 6 Dec. 2014; L. Dányi leg.; (F. 30. slide No. 2624, adult, stained, whole mounted specimen); ELTE.

Paratypes

HUNGARY • 2 specimens; same data as for the holotype (P. 128.1–128.2 slide No. 2598, 2623, adult, stained, whole mounted specimens) • all other paratypes from same type locality; 2 Oct. 2015; D. Angyal, G. Balázs & L. Dányi leg.; P. 128.3–128.5 slide No. 2086, 2643, 2644, three adult, stained, P. 128.6 slide No.2664 only the forepart (with 26 segments) of an adult, stained, whole mounted specimens P.128.7 No. 2638 not stained, whole mounted specimens; P.128.8. slide No. 2639 (body end used for molecular analysis, DNA ID number 911b) not stained, whole mounted specimens; ELTE.

Additional material

HUNGARY • 2 subadult specimens; same type locality; (one of them used for molecular analysis, DNA ID number 911); only *in vivo*.

One adult specimen was studied *in vivo*, stained and whole-mounted. 4 subadult specimens and 2 specimens from type locality, only *in vivo*.

Description

Measurements. Medium-sized, whitish worms. Holotype 9.7 mm long, 370 μm wide at VIII and 380 μm at the clitellum when fixed, 53 segments. Body length of the paratypes 9–15 mm, width 300–390 μm at VIII and 360–400 μm at the clitellum *in vivo*; fixed specimens: length 6.6–11.3 mm, width 310–420 μm at VIII and 310–420 μm at the clitellum, segments 42–61.

Chaetae. Chaetal formula: 2,3,4–4,3,2 : 2,3,4,(5)–4,(5),3,2. Inner chaetae being slightly shorter and thinner than outer: 38–40 × 5 μm and 30–35 × 2,5 μm (in preclitellar bundles). Behind the clitellum from ca. XXVII-XXXV only two chaetae per lateral bundle, posteriorly length about 45–55 × 5 μm. Often detached chaetae in coelom (Fig. 2E).

Head pore. At 0/I longitudinal slit.

Dorsal pores. From VII. Three or four rows of hyaline epidermal gland cells per segment.

Table 1 (opposite page)**.** List of *Fridericia* specimens used for molecular taxonomic analyses with collection data and GenBank accession numbers. Sequences determined in this study appear in bold. Holotypes and paratypes of new species are indicated with H and P in parentheses.

Species	Specimen ID	Locality	Habitat	Reference	GenBank accession numbers		
					ITS	CO1	H3
Fridericia baradlana sp. nov.	911	Hungary, Aggtelek, Baradla cave	bits of decaying wood	this paper	**MK560154**	**MK580963**	**MK562741**
Fridericia baradlana sp. nov.	911b (P)	Hungary, Aggtelek, Baradla cave	bits of decaying wood	this paper	–	–	**MK562742**
Fridericia spelaeophila sp. nov.	1283 (H)	Hungary, Bükk Mts. Kis-kőháti shaft	rotten wood debris	this paper	**MK560155**	**MK580964**	**MK562739**
Fridericia spelaeophila sp. nov.	1284 (P)	Hungary, Bükk Mts. Kis-kőháti shaft	rotten wood debris	this paper	**MK560156**	**MK580965**	**MK562740**
Fridericia dura	907	Hungary, Őrség National Park	on the edge of alder swamp	Dózsa-Farkas & Felföldi 2018, Nagy *et al.* 2018	MF547696	–	KX985894
Fridericia floriformis	1088	South Korea, Jeju Island	clayey soil, meadow	Dózsa-Farkas *et al.* 2019	MH128733	MH124586	MH124597
Fridericia floriformis	1136	South Korea, Jeju Island	clayey soil, meadow	Dózsa-Farkas *et al.* 2019	MH128729	MH124589	MH124598
Fridericia galba	1103	Hungary, Kőszeg Mts	meadow	Nagy *et al.* 2018	MF547697	MF547667	MF547688
Fridericia galba	1123	Hungary, between Szolnok and Szajol	riverine poplar-ash woodlands	Nagy *et al.* 2018	MF547698	MF547668	MF547693
Fridericia glandifera	883	Hungary, near Pilisszentlélek	beech wood	Dózsa-Farkas & Felföldi 2018, Nagy *et al.* 2018	–	MF547671	KX985889
Fridericia hegemon	776	Hungary, Mezőföld	meadow	Nagy *et al.* 2018	–	–	MF547681
Fridericia heliota	CE324	Russia, Krasnoyarsk	lab culture	Erseus *et al.* 2010	–	GU902064	–
Fridericia miraflores	CE801	Sweden, Alingsås	at a lake	Erseus *et al.* 2010	–	GU902074	–
Fridericia ratzeli	844	Hungary, Őrség National Park	hay meadow	Dózsa-Farkas & Felföldi 2018	KX985875	KX985884	KX985895
Fridericia ratzeli	CE782	Sweden, Vårgårda	at a lake	Erseus *et al.* 2010	–	GU902070	–
Fridericia raxiensis	1096	Hungary, Kőszeg Mts	alder carr at creekside	Nagy *et al.* 2018	–	MF547673	MF547685
Fridericia raxiensis	1097	Hungary, Kőszeg Mts	alder carr at creekside	Nagy *et al.* 2018	MF547695	MF547674	MF547686
Fridericia regularis	782	Hungary, Mezőföld	meadow	Nagy *et al.* 2018	MF547703	–	MF547682
Fridericia sohlenii	928	Hungary, Őrség	*Sphagnum* mire	this paper	**MK560157**	–	**MK562743**
Fridericia sohlenii	1045	Hungary, Velem	creekside under *Salix* tree	this paper	**MK560158**	**MK580966**	**MK562744**
Fridericia ventrochaetosa	1102	Hungary, Kőszeg Mts	montane hay meadow	Nagy *et al.* 2018	MF547699	MF547675	MF547687
Fridericia ventrochaetosa	1114	Hungary, Kőszeg Mts	montane hay meadow	Nagy *et al.* 2018	MF547700	MF547676	MF547690
Fridericia waldenstroemi	CE897	Sweden, Strömstad	at a stream	Erseus *et al.* 2010	–	GU902076	–
Hemifridericia parva (outgroup)	511a	Hungary, Kiskunság	open sand steppe oak woodlands	Dózsa-Farkas & Felföldi 2015	KM591939	KM591923	KM591931

Clitellum. In XII–1/2XIII, weakly developed, girdle-shaped, hyalocytes and granulocytes arranged in rows. Body wall about 25–35 μm and cuticle <1 μm thick (Fig. 2F), in forepart slightly stronger than at the body end.

Brain. Egg-shaped, about 160–175 μm long, about 2 times as long as wide *in vivo*; 130–155 μm long and 1.5–2 times as long as wide when fixed (Fig. 2A).

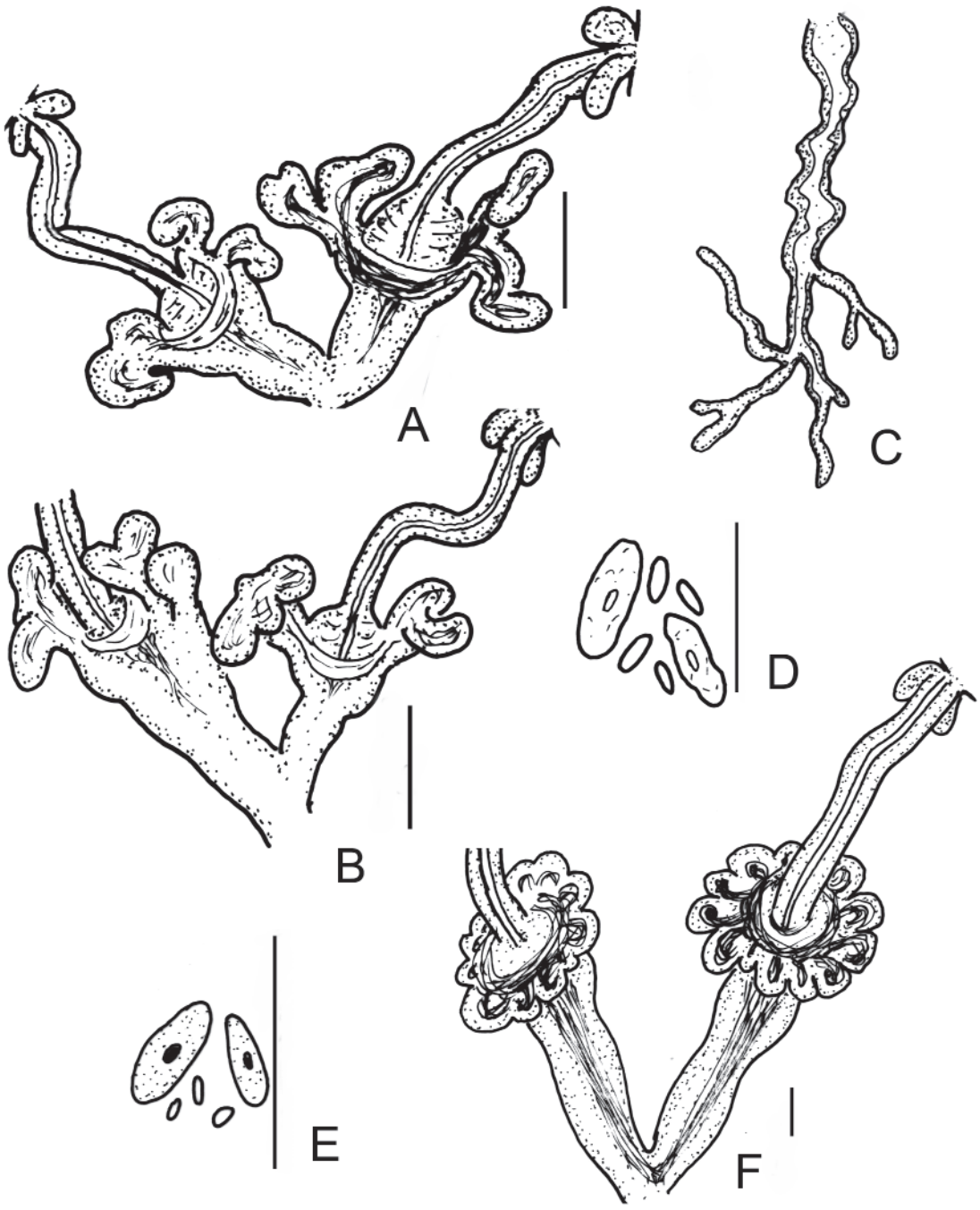

Fig. 1. A–**D**. *Fridericia baradlana* sp. nov. **A**–**B**. Spermathecae. **C**. Oesophageal appendage. **D**. Coelomocytes. **E**–**F**. *Fridericia spelaeophila* sp. nov. **E**. Coelomocytes. **F**. Spermathecae. Scale bars: 50 μm.

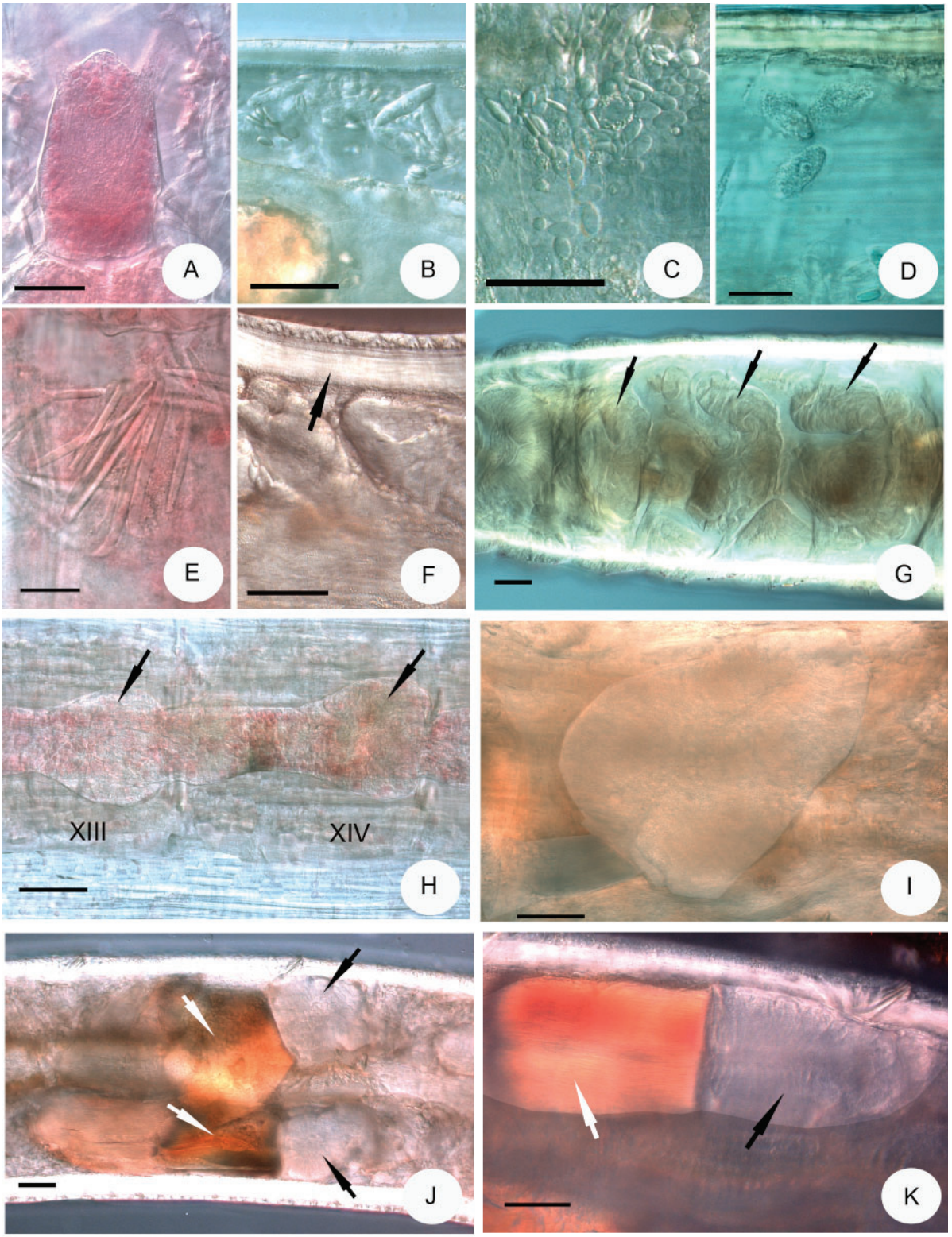

Fig. 2. Micrograph of *Fridericia baradlana* sp. nov. **A**. Brain. **B**–**D**. Coelomocytes (C. several large lenticytes). **E**. Detached chaetae in coelom. **F**. Thick body wall, thin cuticle. **G**. Pharyngeal glands (marked with arrows). **H**. Subneural glands in XIII–XIV (marked with arrows). **I**–**K**. Sperm funnels (funnel's body marked with black arrows, sperm head marked with white arrows). (B–C, F, I–K *in vivo*, A, F, H fixed, stained, D, G fixed, not stained. Scale bars: 50 μm, in D 20 μm. B, E from holotype.)

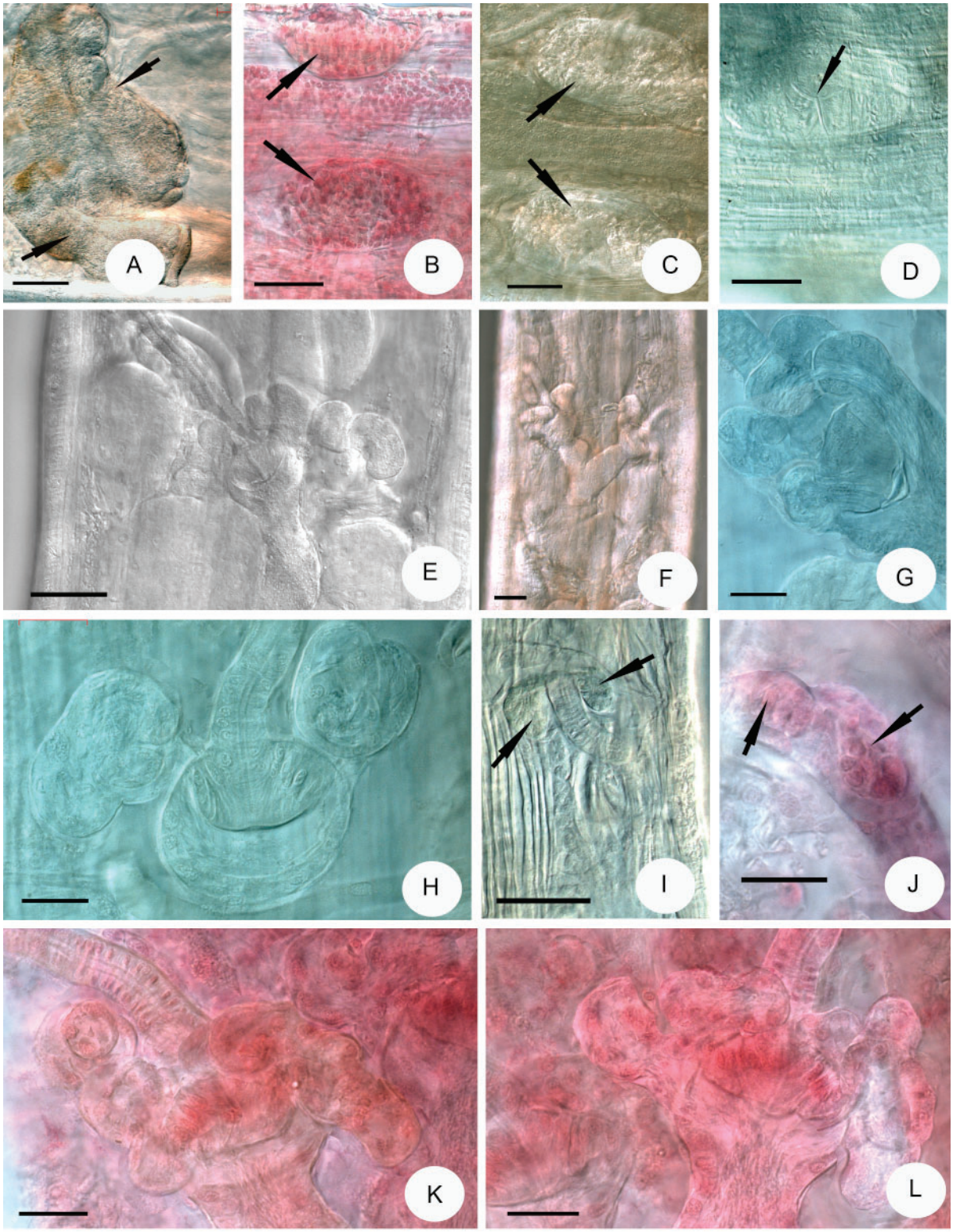

Fig. 3. Micrograph of *Fridericia baradlana* sp. nov. **A.** Sperm funnels (marked with arrows). **B–C.** Male copulatory organs (marked with arrows). **D.** Bursal slit (marked with arrow). **E–H, K–L.** Spermathecae. **I–J.** Spermathecal ectal glands (marked with arrows). (A, C, E–F *in vivo*, B, J, K–L fixed, stained, D, G, H–I fixed, not stained). Scale bars: 50 µm, in H, J–L 20 µm, J–L from holotype.

Oesophageal appendages. Long with many branches at the end in V (Fig. 1C). All pharyngeal glands united dorsally, those in 5/6 and 6/7 with ventral lobes, the third pairs largest (Fig. 2G).

Chloragocytes. From V, 20–23 μm long preclitellarly when fixed. Dorsal vessel from XIX–XXI, blood colourless.

Midgut pars tumida. Not visible.

Nephridia. Five pairs of preclitellar nephridia from 6/7 to 10/11, length ratio anteseptale : postseptale 1 : 1.3–1.6, medial origin of the efferent duct.

Coelomo-mucocytes. Scarce, a/c-type of Möller (1971), elliptic (Figs 1D, 2B, D), length 30–40 μm *in vivo*.

Lenticytes. In large numbers, large 10–15 μm long (Figs 1D, 2C). Chylus cells in XII–XIV, occupying 2 segments.

Seminal vesicle. In XI, not brown. Sperm funnels cylindrical (Figs 2I–K, 3A), about 150–230 μm long and about 1.5–2.3 times as long as wide *in vivo*.

Funnel. Length in fixed specimens 95–150 μm, funnel body 1.2–2 times as long as wide but sometimes wider than long; collar as wide as funnel body.

Spermatozoa. Length: 250–320 μm, heads 110–150 μm *in vivo* (Fig. 2J–K); when fixed 140–170 μm and heads 80–95 μm. Diameter of sperm ducts 7–9 μm *in vivo* (9–10 μm, when fixed).

Male copulatory organs. 115–140 μm long, 70–80 μm wide and 50–59 μm high *in vivo* (Fig. 3C); 70–130 μm long, 50–70 μm wide and 40–60 μm high when fixed (Fig. 3B). Bursal slits T-shaped (Fig. 3D).

Subneural glands. In XIII–XIV (Fig. 2H).

Spermathecae (Figs 1A–B, 3E–H). Two ectal glands of variable length (25–50 μm long) (Figs 3I–J); ectal ducts contractile, thus the length variable, about 150–250 μm long and 16–17 μm wide, canal 2 μm wide *in vivo* (85–185 μm long, 16–17 μm wide when fixed), not widened entally, projecting into ampulla, ental bulbs about 30–40 μm wide when fixed.

Ampullae. With two long, arm-like diverticula, in their proximal parts dividing into an upper and a lower branch and variously bent (not easily visible, since the branches are overlapping with each other). Proximal part of ampullae fused, with common opening into oesophagus dorsally.

Distribution and habitat

Only known from the type locality: Baradla cave, Aggtelek Karst, Hungary.

Differential diagnosis

There are only four valid species of *Fridericia* with two elongate spermathecal diverticula and with the proximal parts of the two spermathecal ampullae fused, forming one common opening into the oesophagus: *F. waldenstroemi* Rota & Healy, 1999, *F. montafonensis* Schmelz, 1998, *F. profundicola* Dózsa-Farkas, 1991, and *F. longeaurita* Boros & Dózsa-Farkas, 2015. The new species differs from all these species (leaving other characters out of consideration) by the form of diverticula, which divide into an upper and a lower finger-like branch from their base at the ampulla.

Fridericia spelaeophila sp. nov.
urn:lsid:zoobank.org:act:FC10F756-F699-4776-B6DC-9C09B8A135CD
Figs 1, 4–5

Diagnosis

The new species can be recognized by the following combination of characters: (1) large size (body length 17–29 mm *in vivo*); segments 48–73; (2) chaetae maximum 5–6 (7) per bundle, many detached chaetae in coelom; (3) clitellum girdle-shaped, weakly developed; (4) body wall thick (40–60 µm) and cuticle thin (1.5–2 µm) *in vivo*; (5) five preclitellar pairs of nephridia; (6) coelomo-mucocytes c-type of Möller (1971), lenticytes 5–10 µm; (7) oesophageal appendages long with some branches at the end; (8) dorsal vessel from 11/12 (peculiar character); (9) seminal vesicle in XI; (10) subneural glands absent; (11) sperm funnel rounded, about ⅓ as long as body diameter, collar about as wide as funnel diameter, spermatozoa 150–170 µm long, heads 50–70 µm when fixed; (12) spermatheca with 10–15 sessile diverticula with sperm in them, ectal duct of variable length with 2–3 large ectal glands of variable size; proximal part of ampullae conspicuous, fused, with common opening into oesophagus dorsally.

Etymology

From the composition of spelaeo (Latin: spēlaeum, i) 'cavern' and phila (Latin: -philus, -phila) 'lover', as it was collected in a cave.

Material examined

Holotype

HUNGARY • Kis-kőháti shaft (Bükk Mts), coarse woody debris; 48°04′05.1″ N, 20°33′07.7″ E; 1 May 2018; L. Dányi & G. Balázs leg. (F.31. slide No. 2607. adult, stained whole-mounted specimen, last 11 segments used for molecular analysis, DNA ID number 1283); ELTE.

Paratypes

HUNGARY • 2 specimens; same data as for the holotype (P. 129.1–129.2 slide No. 2646, 2650, last 19 segments for molecular analysis, DNA ID number 1284).

HUNGARY • 3 specimens; Szepesi-Láner cave system (Bükk Mts): Láner Olivér cave, debris of decaying wood and clay; 48°05'59.8" N, 20°35'41.7" E; 16 Oct. 2014; D. Angyal, G. Balázs & L. Dányi leg. (P. 130.1–130.3 slide No. 2647a+b; 2667a+b; 2085a+b) • 8 specimens; 27 Jan. 2015; D. Angyal, G. Balázs & L. Dányi leg. (P. 130.4–130.11 slide No. 2648; 2649a+b, 2651a+b; 2665a+b; 2668a+b; 2669a+b; 2670a+b; 2676a+b); ELTE.

Additional material

2 juvenile specimens only *in vivo* from Láner Olivér cave. Three adult specimens were fixed on site, but due to the improper fixation these were not suitable for detailed morphological analysis.

Description

Measurements. Large, whitish worms. Holotype 59 segments, 23.5 mm long, 0.75 mm wide at VIII and 0.80 mm at the clitellum *in vivo*; fixed specimen: 12.7 mm long, 0.89 mm wide at VIII and 1.0 mm at the clitellum (later from this specimen, 10 segments were taken for molecular analysis, DNA ID number 1283). Body length of the paratypes 17–27 mm, width 0.60–0.78 mm at VIII and 0.77–0.90 mm at the clitellum *in vivo*; fixed specimens 10–29 mm long, width 0.75–1.2 mm at VIII and 0.90–1.25 mm at the clitellum; segments 48–73.

Chaetae. Chaetal formula: 2,3,4,5,(6,7)–5,4,2 : (2),4,5,6,(7)–(6),4,3,2. Mostly inner chaetae being slightly shorter and thinner than the outer ones: e.g. 50–70 × 5–7 µm and 74–100 × 9–11 µm (Fig. 4C),

but sometimes placed irregularly (e.g. from one side to the other half in line: 78 × 8; 100 × 9; 100 × 9; 75 × 8, 93 × 9 µm) or almost equally long and thick in preclitellar bundles (Fig. 4E). Many detached chaetae in packages in coelomic cavity (Fig. 4F). In these packages the length of largest chaetae was 104 × 8–9 µm, the length of the smallest one 47 × 6 µm. From segment XVI or at the latest from XXII, in the lateral bundles only 2 chaetae, length about 80 × 4.5–7 µm.

Head pore. A longitudinal slit at 0/I (Fig. 4B).

Dorsal pores. From VII. Epidermal gland cells inconspicuous.

Clitellum. In XII–1/2XIII, weakly developed, girdle-shaped, gland cells irregularly arranged (Fig. 4D), between bursal slits mostly granulocytes.

Body wall. Thick, about 35–60 µm, cuticle thin, about 1–2 µm when fixed.

Brain (Fig. 4A). Egg-shaped, about 170 µm long, about 1.2–1.6 times as long as wide (fixed).

Oesophageal appendages. Long, with some distal branches in V. All pharyngeal glands united dorsally and with short ventral lobes.

Chloragocytes. From V. Dorsal vessel from 11/12 (in one case in XII), with large heart-like expansions in XI–VIII, blood colourless.

Chylus cells and midgut pars tumida. Not visible (probably because the intestine was full of wood-fragments and crystals (most probably calcite) in all studied specimens; Fig. 4I).

Nephridia. Five pairs of preclitellar nephridia from 6/7 to 10/11, length ratio anteseptale : postseptale 1 : 1.3–1.4 (Fig. 4H), adseptal origin of efferent duct.

Coelomo-mucocytes. c-type: with smooth outline, elliptic, matrix pale, with well visible nucleoli, 17–26 µm long *in vivo*, 13–25 µm long when fixed (Figs 1E, 4G).

Lenticytes. 5–10 µm long.

Seminal vesicle. In XI or X–XI.

Sperm funnels. Mostly roundish, about 270–300 µm long and about 1.4–1.6 times longer than wide *in vivo* (Fig. 4K). Funnel (Figs 4K–L, 5A) length in fixed specimens 180–300 µm, funnel body 1.2–1.4 times longer than wide, sometimes two times as long as wide; collar about as wide as funnel body (Figs 4L, 5A). Length of spermatozoa not measurable *in vivo*, heads 110–130 µm (Fig. 4K), in fixed specimens, spermatozoa 150–170 µm long, sperm heads 50–70 µm. Diameter of sperm ducts 8–9 µm when fixed.

Male copulatory organs. 100–200 µm long, 90–180 µm wide and about 80 µm high when fixed (Fig. 4J), glandular body weakly developed. Bursal slits T-shaped.

Subneural glands. Absent.

Spermathecae (Figs 1F, 5B–H). Two or three large ectal glands, size variable (35–97 µm long *in vivo* and 40–90 µm long, fixed); ectal ducts about 310–340 µm long and 37–38 µm wide, canal 8–8.5 µm wide *in vivo* (when fixed, variable: 150–300 µm long, 27–40 µm wide, canal 6–8 µm), not or slightly widened entally, projecting into ampulla, ental bulbs about 70–84 µm wide when fixed.

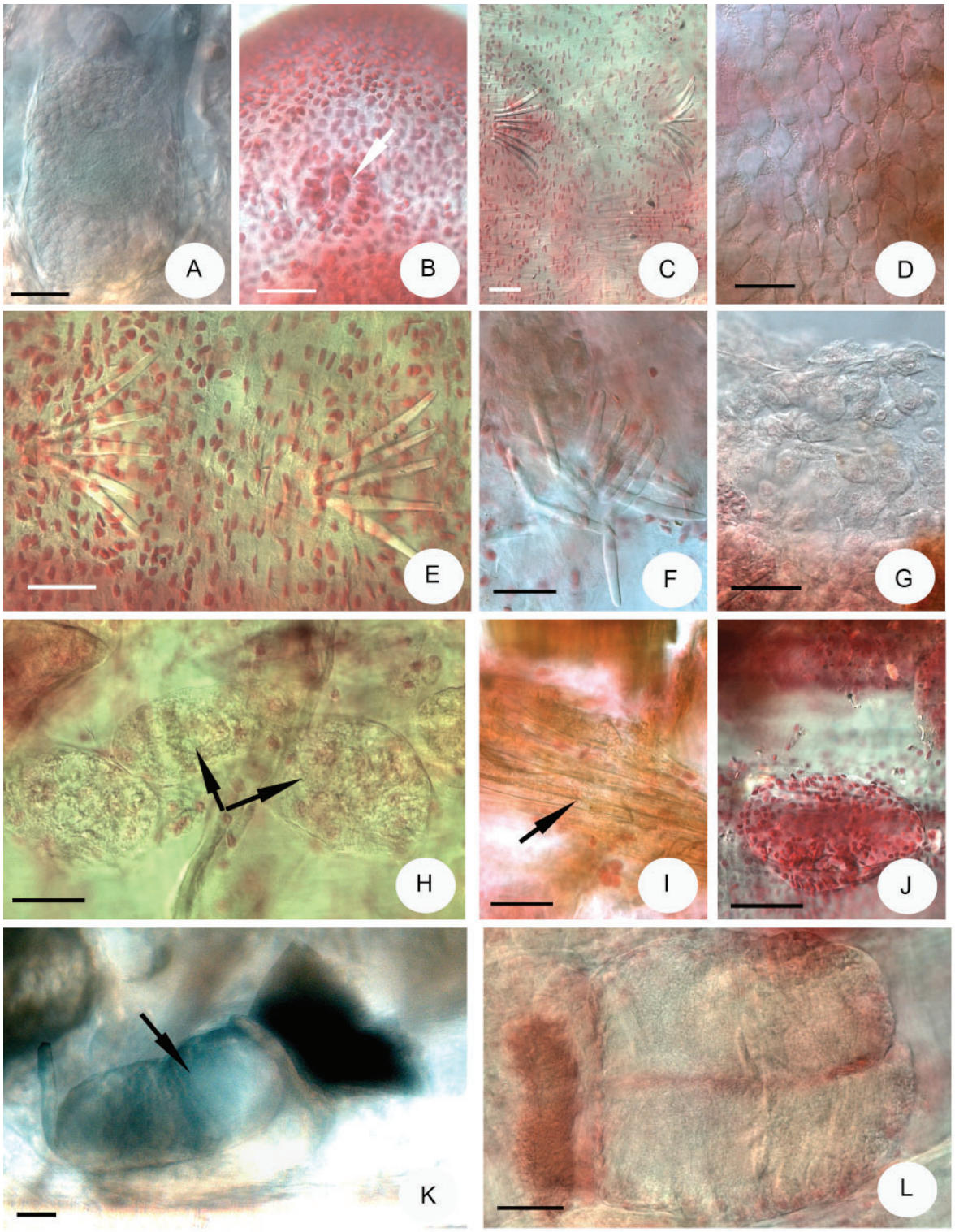

Fig. 4. Micrograph of *Fridericia spelaeophila* sp. nov. **A**. Brain. **B**. Head pore (marked with arrow). **C**. Chaetae in V–VII. **D**. Clitellar glands, dorsal view. **E**. Chaetae in VII–VIII. **F**. Detached chaetae in coelom. **G**. Coelomocytes. **H**. Nephridium at 7/8 (pre- and postseptale marked with arrows). **I**. Fragments of decaying wood in the intestine (marked with arrow). **J**. Male copulatory organs, lateral view. **K**–**L**. Sperm funnels. (A, K *in vivo*, B–J, L fixed, stained. Scale bars: 50 µm. B, G from holotype, K from DNA 1283.)

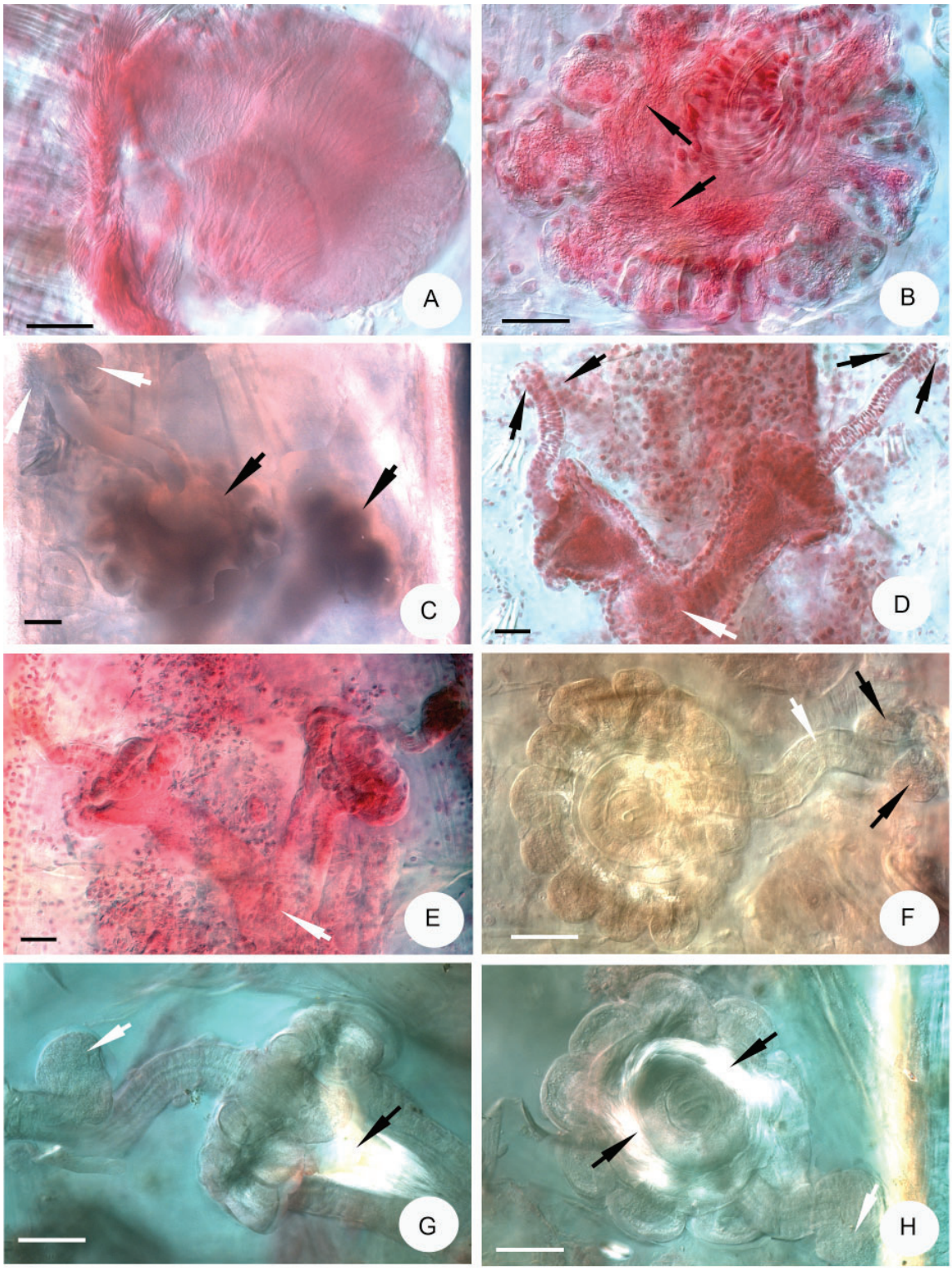

Fig. 5. Micrograph of *Fridericia spelaeophila* sp. nov. **A**. Sperm funnel. **B**, **H**. Spermathecal ampullae (sperm in ampulla marked with arrows). **C–G**. Spermathecae. (In C, diverticula marked with black arrows, ectal glands with white arrow. D–E. Fused proximal parts marked with white arrows, ectal glands with black arrows. F. Ectal duct marked with white arrow, ectal glands with black arrows. G. Ectal glands marked with white arrow, the refractive sperm in ampullar distal part marked with black arrow). All pictures fixed and stained. E from holotype. Scale bars: 50 μm.

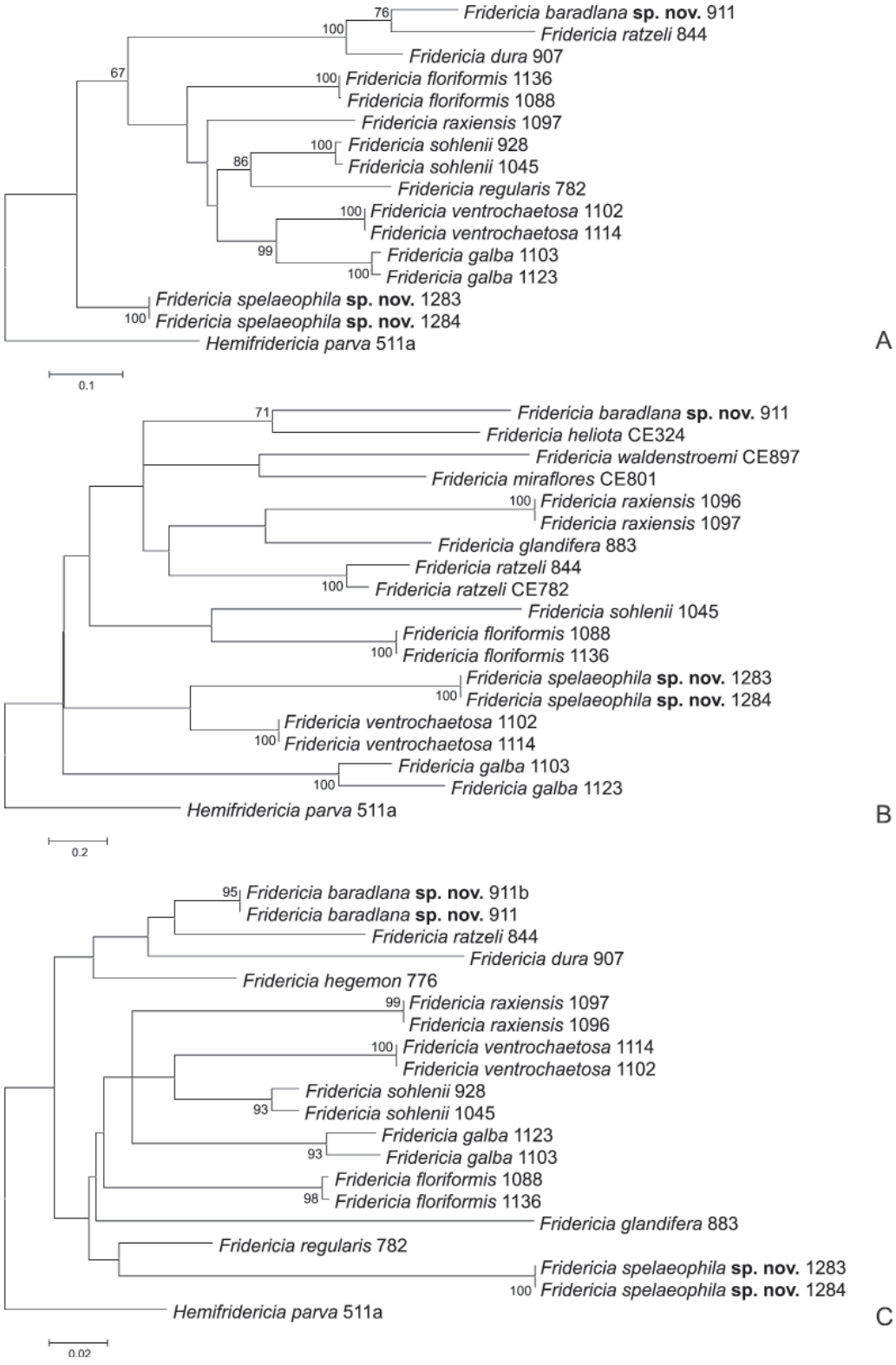

Fig. 6. Maximum likelihood (ML) trees of studied *Fridericia* species based on the ITS region (A), CO1 (B) and H3 genes (C). Bootstrap values greater than 50 are shown at the nodes. Sequences from new species described here appear in bold. **A**. ML tree of the ITS region based on 821 nucleotide positions. **B**. ML tree of the CO1 gene based on 388 nucleotide positions. **C**. ML tree of the H3 gene based on 202 nucleotide positions.

Ampullae. Surrounded distally by about 10–15 sessile diverticula (Figs 5B, F, G–H), these diverticula are 50–90 μm long and 40–90 μm wide. Sperm in a circle in lumen of ampullar distal part and also in the diverticula (Figs 5B, G–H). Diameter of ampulla and diverticula together 180–300 μm when fixed. Proximal part of ampullae conspicuous, 100–320 μm long when fixed and fused into one common duct, which opens into oesophagus dorsally (Figs 5D–E). Often the length of two the proximal part is different (e.g. 130 μm and 255 μm long). 1–2 rather small mature eggs at a time.

Distribution and habitat

In Kis-kőháti shaft and Szepesi-Láner cave system, in decaying wood, Bükk Mts, Hungary.

Differential diagnosis

The new species differs from all *Fridericia* species described up to now by the unusual origin of the dorsal vessel before clitellar segments (mostly in XI from septum 11/12, only in one specimen in XII). Only in the following species of *Fridericia* does the dorsal vessel originate in XIII (intraclitellarly) or XIV, but not preclitellarly: *F. parasitica* Černosvitov, 1928, *F. pretoriana* Stephenson, 1930, *F. monochaeta* Rota, 1995, *F sousai* Schmelz, 2013 and *F. cusanica* Schmelz, 2003. Other differences: *F. sousai* and *F. monochaeta* are smaller (38–43 and 30–35 segments) and have only two spermathecal diverticula; *F. parasitica* has 40–52 segments but only 4 spermathecal diverticula; *F. pretoriana* has 36–51 segments and *F. cusanica* 32–35 segments and spermatheca without diverticula.

Results of molecular analysis

In total, 5, 4 and 6 new sequences were obtained from the studied *Fridericia* species in the case of ITS, CO1 and H3, respectively. Unfortunately, we failed to amplify the ITS region and CO1 gene from some specimens, which was probably due to the improper hybridization of PCR primer sequences to the extracted genomic DNA. Results of molecular analysis confirmed that *Fridericia baradlana* sp. nov. and *Fridericia spelaeophila* sp. nov. are distinct species, since based on the three studied regions, sequences acquired from the examined specimens were clearly separated in the phylogenetic trees (Figs 6–7).

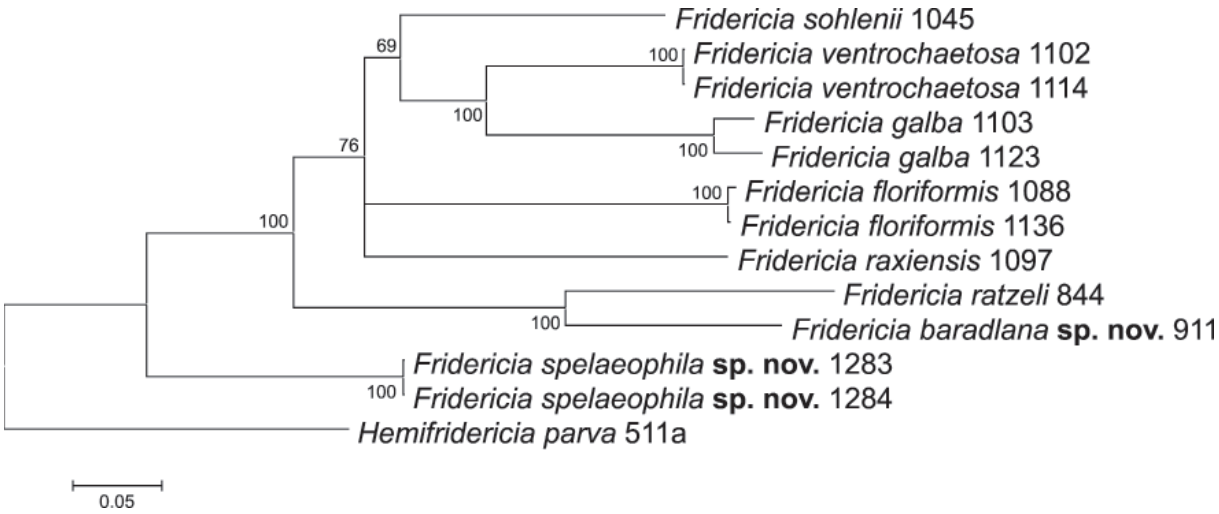

Fig. 7. Concatenated phylogenetic tree of the two new and other *Fridericia* species based on 1447 nucleotid positions (which comprise the CO1, H3 genes and the ITS region). Posterior probabilities greater than 50 are shown at the nodes. Sequences from new species described here appear in bold.

Discussion

In Hungary, enchytraeids have been reported from three caves so far: 17 species from the Baradla cave in the Aggtelek Karst (Dózsa-Farkas 1970, 1974), 3 species from the Meteor cave in the Bükk Mts (Bajomi 1969) and 4 species from the Szemlő-hegyi aragonite cave near Budapest (Dózsa-Farkas 1989). Most of the recorded species are widespread and common, and none of them can be considered troglobiotic.

From the two new species, *F. spelaeophila* sp. nov. (which has been detected in two localities) surely prefers habitats with higher moisture, and it has been found up to now only in a cave and a shaft (its name alludes to this), but we cannot state that it is a troglobiotic species. The troglobiotic, endemic status of *F. baradlana* sp. nov. is also not sure, although it has been only recently discovered in the intensively explored Baradla cave and was not recorded at any other site. Based on their intestinal content, specimens of both species found appropriate food in the decaying wood.

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