EXTENDING THE GEOGRAPHIC DISTRIBUTION OF *BRYODRILUS EHLERSI* (ANNELIDA, ENCHYTRAEIDAE): MORPHOLOGICAL AND MOLECULAR COMPARISON OF KOREAN AND EUROPEAN SPECIMENS

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During the study of the enchytraeid fauna of South Korea, specimens were found, which were identified as *Bryodrilus ehlersi* based on their morphological characters. This was an unexpected result since this species is common in Northern and Central Europe but has not yet been found in the Far East. Furthermore, sequence analysis of the mitochondrial cytochrome c oxidase subunit I gene, the nuclear histone 3 gene and the nuclear ribosomal ITS region showed unambiguously that the specimens collected in South Korea represent a different evolutionary lineage. In this paper, the detailed morphological and molecular comparison of *Bryodrilus ehlersi* specimens collected from various sites and countries are presented. Although the Korean specimens could not be formally described as a different species due to the lack of studied specimens from the type locality, clear evidence of cryptic genetic diversity was found within *Bryodrilus ehlersi*.

Key words: Enchytraeidae, cryptic diversity, *Bryodrilus ehlersi*, South Korea, molecular taxonomy.

INTRODUCTION

Molecular techniques that supplement morphological methods have become extremely important in taxonomic studies. There are so-called "cryptic species", that differ genetically but their morphology is very similar, therefore cannot or can only be hardly differentiated with traditional morphological methods, and they can be identified and distinguished only by means of molecular analyses (Schmelz *et al.* 2017, and references cited therein). In the Enchytraeidae, several new cryptic species have already been described, e.g.,

Enchytraeus crypticus Westheide et Graefe, 1992, *Cognettia pseudosphagnetorum* Martinsson *et al.*, 2015 and *C. varisetosus* Martinsson *et al.*, 2015 and *C. asloae* Martinsson *et al.* 2018. According to Schmelz *et al.* (2017) and Martinsson *et al.* (2018), if two species are morphologically hardly distinguishable or indistinguishable, they can be considered as cryptic species pairs [e.g., *C. sphagnetorum* (Vejdovský, 1878) and *C. pseudosphagnetorum*]. Sometimes, DNA-based studies have aided the recognition of distinct evolutionary lineages within the same morphospecies, but the formal description of a new species is yet to be done, e.g., in the case of specimens morphologically identified as *Achaeta danica* (Dózsa-Farkas & Felföldi 2017), *Cernosvitoviella minor* (Dózsa-Farkas *et al.* 2017) and *Fridericia seoraksani* (Dózsa-Farkas *et al.* 2015). Furthermore, clear evidence of cryptic diversity was also found within the genus *Marionina* (Matamoros *et al.* 2012) and, more recently, in *Globulidrilus*, *Hemifridericia* and *Stercutus* (Martinsson & Erséus 2018).

Molecular methods provide an opportunity to verify the identity of morphologically slightly different enchytraeids previously described as separate species and whether these dissimilarities should be considered as intraspecific variations and synonymization should be performed. In the case of the *Bryodrilus* genus, which is the subject of this study, this has already been done when *Bryodrilus parvus* Nurminen, 1970 was synonymised with *B. librus* (Nielsen & Christensen, 1959) (Dózsa-Farkas *et al.* 2012), and *Bryodrilus glandulosus* Dózsa-Farkas, 1990 was synonymised with *Bryodrilus ehlersi* Ude, 1892 (Cech *et al.* 2012).

A suspected case of cryptic species was observed in *Bryodrilus ehlersi* Ude, 1892 s.l. when comparing specimens from South Korea with those collected from the previously known distribution area of this species (northern and central regions of Europe). Our ongoing research project on the enchytraeid fauna of South Korea is based on soil samples collected in 2016 and 2017, and specimens have been identified, as suggested by Schmelz *et al.* (2017), using a combination of morphological and molecular analyses. Taxonomic and faunistic results have been partly published with the description of twelve species and one genus new to science (Dózsa-Farkas *et al.* 2018, 2019*a,b*), while in this paper, based on specimens from this sampling campaign, the comparison of Korean and European *Bryodrilus ehlersi* specimens is given.

MATERIAL AND METHODS

Abbreviations: cf. = confer, CO1 = Cytochrome c Oxidase subunit I, H3 = Histone 3, ITS = Internal Transcribed Spacer, PCR = Polymerase Chain Reaction. Institutional abbreviations: NIBRIV = National Institute of Biological Resources, Korea

Morphological methods – Specimens were collected in South Korea (Mt. Deogyu and Mt. Odaesan), Germany and Hungary (see details in Table 1 and in the species description). Worms were extracted from soil by the wet funnel method (O'Connor 1962). Enchytraeids

were first observed and measured alive, then fixed and preserved in 70% ethanol for DNA-based analysis. Some of the specimens 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). All the important morphological characters were recorded *in vivo*, drawn and photographed [Axio Imager.A2 microscope with differential interference contrast illumination, AxioCam MRc 5 (Zeiss) digital camera, Axiovision software]. The whole-mounted specimens were reinvestigated and photographed as well. In all micrographs presented in this study, the orientation of specimens is the same: the head is either on the left side or on the top of the picture. Reference material of selected studied specimens was deposited in the National Institute of Biological Resources, Korea ("NIBRIV").

DNA-based methods – Genomic DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen) according to the instructions given by the manufacturer. CO1 and H3 genes and the ITS region were amplified separately by 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. PCRs and sequencing 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) and by the Biomi Ltd. (Gödöllő, Hungary), while the maximum likelihood analyses including the search for the best-fit model were performed with the MEGA 7 software (Kumar *et al.* 2016). Additionally, a concatenated analysis with discrete substitution models was performed. For this, 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 obtained sequences were deposited in GenBank under the following accession codes: MK098303-MK098308 (H3), MK098309-MK098314 (CO1) and MK108074-MK108077 (ITS).

RESULTS

Morphological characterization of the Korean Bryodrilus ehlersi *specimens*

Korean material deposited – In total, 14 stained, adult specimens on slides. NIBRIV0000831786, slide No. 2675, adult, stained, whole mounted specimen, Mt. Deogyu, Seolcheon-meyon, Muju-gun, Jeollabuk-do, South Korea, decaying wood debris, soil and litter layers, 35°51′39.9″N, 127°45′22.9″E, 1245 m a.s.l., 21.09.2016, Leg. Yong Hong. NIBRIV0000831787, slide No. 2411 (DNA ID 1078), NIBRIV0000831788, slide No. 2632., P.124.1.1-124.1.9, slides No. 2465–2466, 2579–2580, 2633, 2671–2674, 11 specimens, same data as above. P.124.11–124.12, slides No. 2604, 2631 (DNA ID 1295), 2 specimens, Waljeong-sa, Mt. Odaesan, Dongsan-ri, Jinbu-myeon, Pyeongchang-gun, Gangwon-do, South Korea, decaying wood debris, soil and litter layers in *Abies holophylla* forest, 37°43′36.9″N, 128°35′47.5″E, 630 m a.s.l., 01.11.2017, Leg. Yong Hong.

Table 1. List of *Bryodrilus* specimens used for molecular taxonomic analyses with collection data and GenBank accession numbers. Sequences generated in this study appear in bold, n.d. – no data.

Species	Specimen ID	Locality	Habitat	Reference	ITS	CO1	H3
						GenBank accession number	
B. cf. *ehlersi*	1078	S Korea, Mt. Deogyu	decaying wood debris, soil	this paper	**MK108075**	**MK098309**	**MK098303**
B. cf. *ehlersi*	1079	S Korea, Mt. Deogyu	decaying wood debris, soil	this paper	**MK108076**	**MK098310**	**MK098304**
B. cf. *ehlersi*	1162	S Korea, Mt. Deogyu	decaying wood debris, soil	this paper	–	**MK098311**	**MK098305**
B. cf. *ehlersi*	1295	S Korea, Mt. Odaesan	decaying wood debris, soil of *Abies holophylla* forest	this paper	**MK108074**	**MK098312**	**MK098306**
B. diverticulatus	brydiv-svalbard9	Svalbard, Russebukta	coastal moss tundra	Dózsa-Farkas *et al.* 2012, Ávila-Jiménez *et al.* 2019	–	JN641202	–
B. diverticulatus	brydiv-svalbard11	Svalbard, Kapp Lee	valley floor moss tundra	Dózsa-Farkas *et al.* 2012, Ávila-Jiménez *et al.* 2019	–	JN641203	
B. diverticulatus	brydiv-svalbard29	Svalbard, Blåbukta	terraced moss tundra	Dózsa-Farkas *et al.* 2012, Ávila-Jiménez *et al.* 2019	–	JN641201	
B. diverticulatus	CE19814	Norway, Engerdal	n.d.	Martinsson *et al.* 2017	–	KX618735	–
B. ehlersi	105	Hungary, Mt Villány	decaying bark on tree stump	Cech *et al.* 2012	JF750404		
B. ehlersi	176	Germany, Lake Stamberg	mixed deciduous forest	Cech *et al.* 2012	JF750405	–	–
B. ehlersi	203	Hungary, Mt. Bakony	decaying bark on tree stump	Cech *et al.* 2012	JF750407	–	–
B. ehlersi	238	Hungary, Mt. Gerecse	decaying bark on tree stump	Cech *et al.* 2012	JF750408	–	–
B. ehlersi	bryehl-bank	Hungary, Mt. Bakony	decaying bark on tree stump	Dózsa-Farkas *et al.* 2012	–	JN641204	–

Table 1 (continued)

Species	Specimen ID	Locality	Habitat	Reference	GenBank accession number		
					ITS	CO1	H3
B. ehlersi	1112	Hungary, Kőszeg Mts	alder carr	this paper	–	**MK098313**	**MK098307**
B. ehlersi	1113	Hungary, Kőszeg Mts	alder carr	this paper	**MK108077**	**MK098314**	**MK098308**
B. hallasanensis	994	S Korea, Mt. Hallasan	soil of *Sorbus commixta* tree	Dózsa-Farkas *et al.* 2018	–	MG252133	MG252175
B. hallasanensis	1018	S Korea, Mt. Hallasan	soil of grass	Dózsa-Farkas *et al.* 2018	MG252201	MG252134	MG252176
B. hallasanensis	1092	S Korea, Mt. Hallasan	soil and moss layers of *Sorbus alnifolia* forest	Dózsa-Farkas *et al.* 2018	MG252202	MG252135	–
B. hallasanensis	1130	S Korea, Mt. Hallasan	soil of grass	Dózsa-Farkas *et al.* 2018	MG252203	MG252136	MG252177
Henlea ochracea (outgroup)	249	Alaska, Chena Bridge	mixed forest	Dózsa-Farkas *et al.* 2015	KR872382	KR872329	KR872361

Additional material examined from Korea – 4 subadult specimens and 2 specimens only alive from the type locality.

Comparative material from Europe – Hungary: 2 specimens, Kőszeg Mts, near Paprét, alder carr at a creekside, 47°24′05.5″N 16°26′52.7″E, 478 m a.s.l., 21.05. 2014, Leg. Klára Dózsa-Farkas, János Farkas, Zoltán Tóth (DNA ID 1112 and 1113); 10 specimens, Börzsöny Mts, Királyrét, decaying bark on tree stumps, 47°56′14.6″N 18°56′26.2″E, 774 m a.s.l., 17.10.2017, Leg. János Farkas; 1 specimen, Mt. Villány, Szársomlyó, oak forest with *Ruscus aculeatus*, decaying bark on tree stump, 45°51′25″N, 18°25′13″E, 313 m a.s.l., 04.04.2006, Leg. Klára Dózsa-Farkas, János Farkas, Gergely Boros (DNA ID 105); 1 specimen, Mt. Bakony, Bakonybél - Pápa, beech forest, decaying bark on tree stump, 47°17′27″N 17°42′20″E, 285 m a.s.l., 17.07.2008, Leg. Klára Dózsa-Farkas, Jenő Kontschán, Balázs Németh (DNA ID 203); 1 specimen, Mt. Bakony, beech forest, decaying bark on tree stump, 47°06′42″N 17°47′19″E, 370 m a.s.l., 05.01.2011, Leg. Klára Dózsa-Farkas, Jenő Kontschán (DNA ID bryehl-bank); 1 specimen, Mt. Gerecse, Tardos, mixed deciduous forest, decaying bark on tree stump, 47°40′15″N 18°26′42″E, 270 m a.s.l., 24.11.2009, Leg. Klára Dózsa-Farkas, Jenő Kontschán (DNA ID 238). Germany: 1 specimen, Lake Stanberg, mixed deciduous forest, 48°00′N 11°22′E, 10.03.2008, Leg. Klára Dózsa-Farkas, András Dózsa-Farkas (DNA ID 176). See additional details in Cech *et al.* (2012) and Dózsa-Farkas *et al.* (2012).

Short description of the Korean specimens – The following combination of characters: (1) middle size (11–20 mm *in vivo*), segments 38–52; (2) chaetae maximum 6–7 (8) per bundle, sigmoid without nodulus; (3) clitellum girdle shaped; (4) seven pairs of preclitellar nephridia; (5) coelomocytes discoid; (6) pharyngeal glands free dorsally and with ventral lobes; (7) two pairs of oesophageal appendages in VI with sponge-like structure not pulsating in living worms; (8) sperm funnels pear-shaped or cylindrical, 70–120 µm long and 1.2–2.0 times longer than wide *in vivo*; (9) midgut pars tumida XXIII–XXIX, occupying 3–6 segments; (10) male copulatory organs spherical, diameter about 50–70 µm, fixed; (11) spermathecae communicating with oesophagus, ectal ducts 110–180 µm long and 20–25 µm wide, the ampullae lemon-shaped, diameter 30–50 µm, ectal gland stalked, 30–50 µm long fixed.

Detailed description of the Korean specimens – Middle sized worms. Length 11.0–19.8 mm, width 310–610 µm at VIII and 350–690 µm at clitellum *in vivo*; fixed specimens: length 5.1–16.3 mm, width 410–570 µm at VIII and 420–600 µm at clitellum; segments 38–52. Chaetae sigmoid, without nodulus. Chaetae in a bundle arranged in asymmetric fan, of unequal size, those towards dorsal and ventral midlines of body gradually smaller; largest ventral chaetae in a bundle preclitellarly 53–62 µm, the shortest 30–37 µm long and 5–3 µm wide. In terminal segments the largest chaetae 70–80 µm long. Chaetal formula (2),3,4,5 – 3,4: (2,3),4,5,6,7,(8) – 6,5,4,3 (2). Chaetae absent at XII. Three or four rows of epidermal gland cells per segment (Fig. 1D). Head pore at 0/1, a transversal slit (Fig. 1C). Body wall 28–40 µm thick, cuticle thin, 1.0–1.5 µm. Clitellum over XII–1/2 XIII, girdle-shaped, small hyalocytes and granulocytes in irregular rows dorsally and ventrally arranged alike (Figs 1E–G).

Brain 165–220 µm long and 1.3–2.0 times longer than wide *in vivo* (Fig. 1A), 125–160 µm long and 1.1–1.6 times longer than wide when fixed (Fig. 1B), anteriorly convex, posteriorly straight. Pharyngeal glands 3 pairs in IV–VI, most often all free dorsally with ventral lobes (Figs 1I, K, 2J, K), sometimes first pair connected dorsally. Two pairs of oesophageal appendages in VI (Figs 1I–K) with sponge-like structure not pulsating in living worms (about 125–180 µm long *in vivo*, 80–125 µm long, fixed). Seven pairs of preclitellar nephridia from 4/5 to 10/11; anteseptale consisting of funnel and some coil of canal, efferent ducts arising antero-ventrally. Coelomocytes nucleate, discoid, about 17–28 µm long *in vivo* (16–25 µm, fixed), finely granulated, light brown in transmitted light *in vivo* (Figs 1I–J) and with clearly visible nucleus in fixed worms (Fig. 1H). Dorsal blood vessel from XII, blood colourless. Anterior bifurcation in peristomium. The part of midgut in XXIII–XXIX, 3–6 segments long with distinctive hyaline and granulated networking cell structure in the intestinal wall, is different from the structure of other parts of the intestine (Fig. 1M). Chloragocytes from VI, about 25–50 µm large *in vivo*. Intestine filled with decaying wood debris (Fig. 1L). Seminal vesicle small in XI or absent. Sperm funnels (Figs 2C–G), pear-shaped or cylindrical 70–120 µm long and 1.2–2.0 times longer than wide *in vivo* (60–95 µm long and 1.1–1.6 times longer than wide in fixed specimens). Collars about as wide as funnel body or slightly narrower. Diameter of sperm ducts 6–9 µm. Spermatozoa 70–130 µm long, heads 30–45 µm *in vivo* (Fig. 2E), (50–75 µm and 25–30 µm, respectively, when fixed). Male glandular bulbs about 85–110 µm long and 50–80 µm wide *in vivo* (48–70 µm long and 50–70 µm wide when fixed) (Figs 1G, 2B). Bursal slits longitudinal, slightly bent laterally (Fig. 2A). Subneural glands absent. Spermathecal ectal ducts about 135–180 µm long and 20–25 µm wide *in vivo* (110–180 µm long and 20–25 µm wide when fixed), one or two stalked ectal glands 30–50 µm long (Figs 2L–M). Ampullae lemon-shaped, diameter 30–50 µm when fixed. The ental ducts merging entally and with joint opening into oesophagus in VI (Figs 2H–K). 1–2 mature egg at a time.
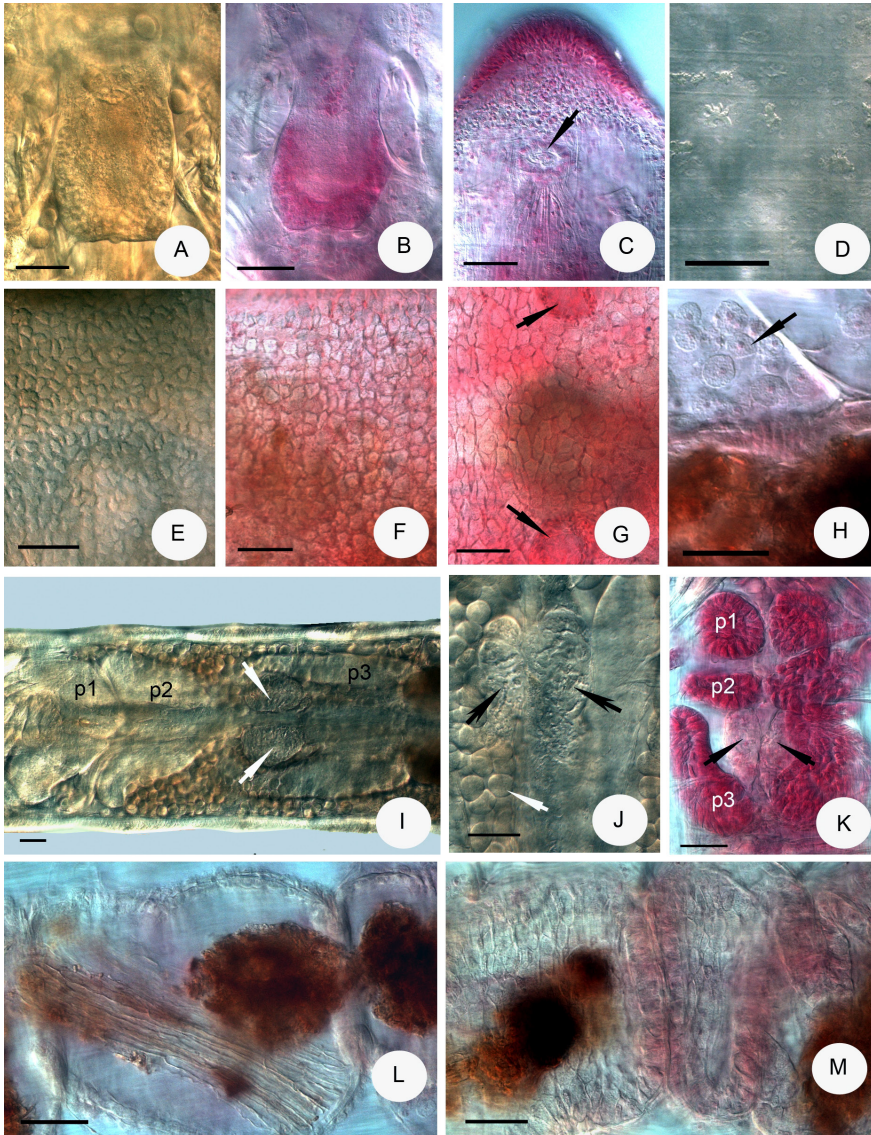
Fig. 1. Micrograph of Korean *Bryodrilus* cf. *ehlersi*. A–B = Brain. C = Head pore (marked with arrow). D = Epidermal gland cells. E–F = Clitellar gland cells, dorsal view. G = Clitellar gland cells, ventral view (penial bulbs marked with arrows). H = Coelomocytes (marked with arrow). I = Segments IV–VI, dorsal view (p1–p3 = pharyngeal glands, oesophageal appendages marked with arrows, coelom full with brown round coelomocytes). J = Oesophageal appendages, dorsal view (marked with black arrows, coelomocytes marked with white arrow). K = Pharyngeal glands (= p1–p3, oesophageal appendages marked with arrows). L = Decaying wood debris in intestine. M = Midgut in XXIII–XXIX. (A, D–E, I–J *in vivo*; B–C, F–H, K–M fixed, stained. Scale bars: 50 μm)
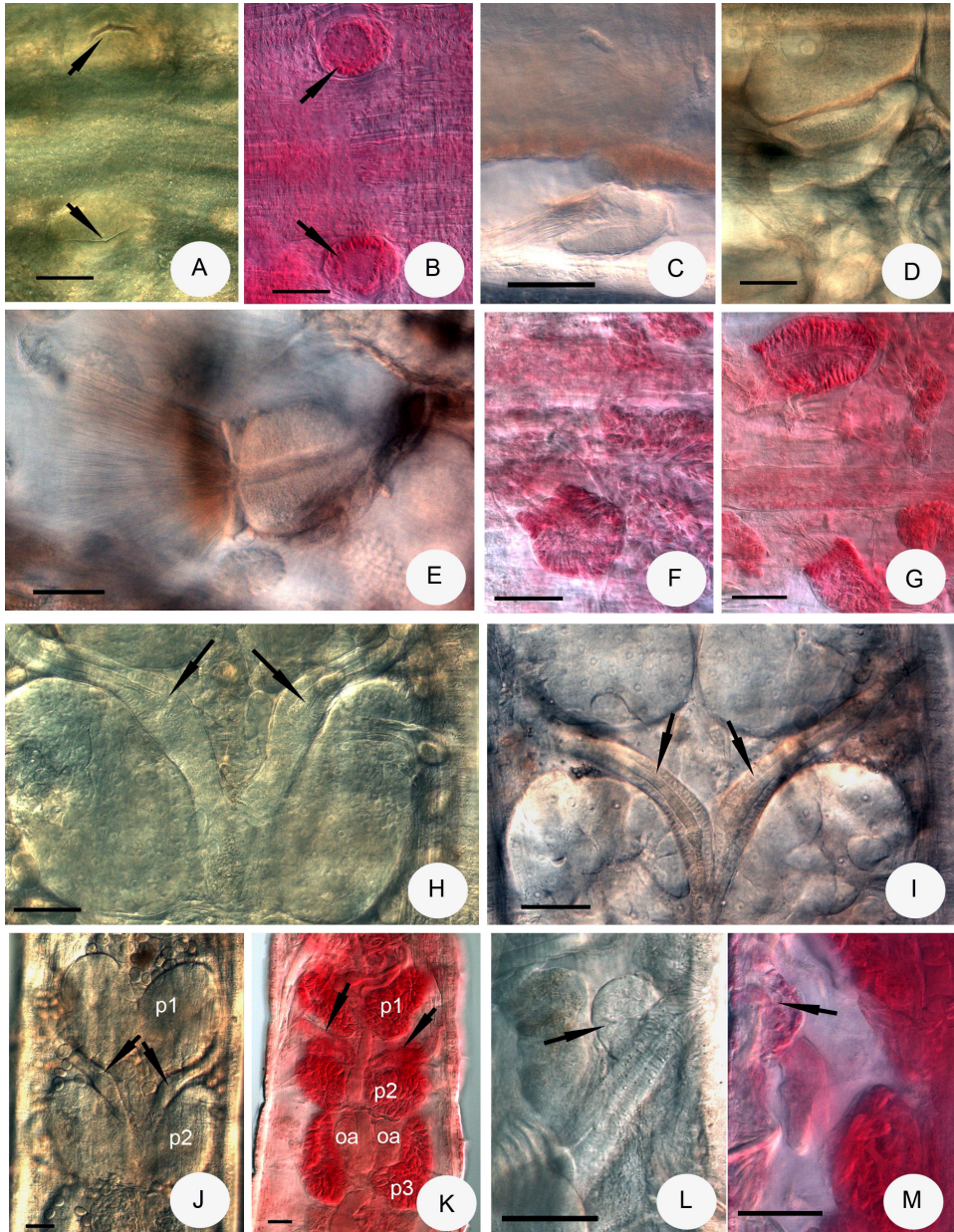
Fig. 2. Micrograph of Korean *Bryodrilus* cf. *ehlersi*. A = Bursal slits, ventral view (marked with arrows). B = Male copulatory apparate (penial bulbs, marked with arrows). C–G = Sperm funnels. H–K = Spermathecae, dorsal view (marked with arrows, p1–p3 = pharyngeal glands). L–M = Ectal glands of spermathecae, lateral view (marked with arrows). (A, C–E, H–J, L *in vivo*; B, F–G, K, M fixed, stained. Scale bars: 50 μm)

Table 2. Comparison of morphological traits of *Bryodrilus ehlersi* specimens collected from Europe and Korea. Abbreviations: BE = *B. ehlersi* (Europe); BK = *B.* cf. *ehlersi* (Korea).

	BE	BK	BE	BK
	in vivo		fixed	
Segments (number)	40–53	38–52		
Length (mm)	9.5–21.0	11.0–19.8	6.0–10.0	5.1–9.2
Width at VIII (µm)	400–600	310–610	400–520	410–570
Width at XII (µm)	450–700	350–690	410–600	420–600
Maximum number of chaetae	6 (7–8)	6–7 (8)		
Length of coelomocytes (µm)	15–30	17–28	13–28	16–25
Length : width of brain	1.6–1.9	1.3–2.0	1.4–1.7	1.1–1.6
Headpore	0/1	transverse	0/1	transverse
No. of nephridia preclitellarly	7	7		
Length of oesophageal appendage (µm)	120–165	125–180	71–119	80–125
Length of sperm funnel (µm)	73–125	45–120	50–150	60–95
Length : width of sperm funnel	1.2-2.3	1.2–2.0	1.2–2.8	1.1–1.6
Lenght of spermatozoa (µm)	82–120	70–130	50–90	50–105
Heads of spermatozoa (µm)	30–40	30–45	25–30	25–40
Length of penial bulb (µm)	60–84	85–110	55–80	48–70
Width of penial bulb (µm)	50–60	50–80	55–68	50–70
Height of penial bulb (µm)	40–47	30–40	55–65	32–40
Length of spermathecal duct (µm)	140–200	135–180	130–180	110–180
Width of spermathecal duct (µm)	21–28	20–25	20–23	20–25
Diameter of ampulla (µm)	30–47	33–40	40–50	30–50
Length of ectal glands (µm)	40–60	43–65	35–60	30–50
Origin of dorsal blood vessel	XII	XII		
Body wall (µm)	23–45	29–32	32–34	28–40
Cuticle (µm)	<1	1	<1	1–1.5

Remarks – The morphological characters of the Korean specimens agree with those of *Bryodrilus ehlersi*, a species common in Northern and Central Europe. The following external and internal characters are essentially the same: size, length, diameter (*in vivo* and fixed alike) and segment number, maximum number of chaetae (6–7), number of nephridia preclitellarly (7 pairs), length of spermatozoa and sperm heads, origin of dorsal blood vessel (in XII), etc. (Table 2). Very conspicuous is the similarity of the oesophageal appendages (sponge-like structure not pulsating), which was exclusive for *B. ehlersi* so far in the genus (Figs 1I–K vs. Figs 3F–H). Further similar morphological features

are: the position of head pore (Fig. 1C vs. Fig. 3B), the shape and size of brain (Figs 1A–B vs. Fig. 3A); the girdle-shaped clitellum, hyaline and granular gland cells mostly in irregular rows dorsally and ventrally (Figs 1E–G vs. Figs 3C–D, J); all three pharyngeal glands with ventral lobes and free dorsally (Figs
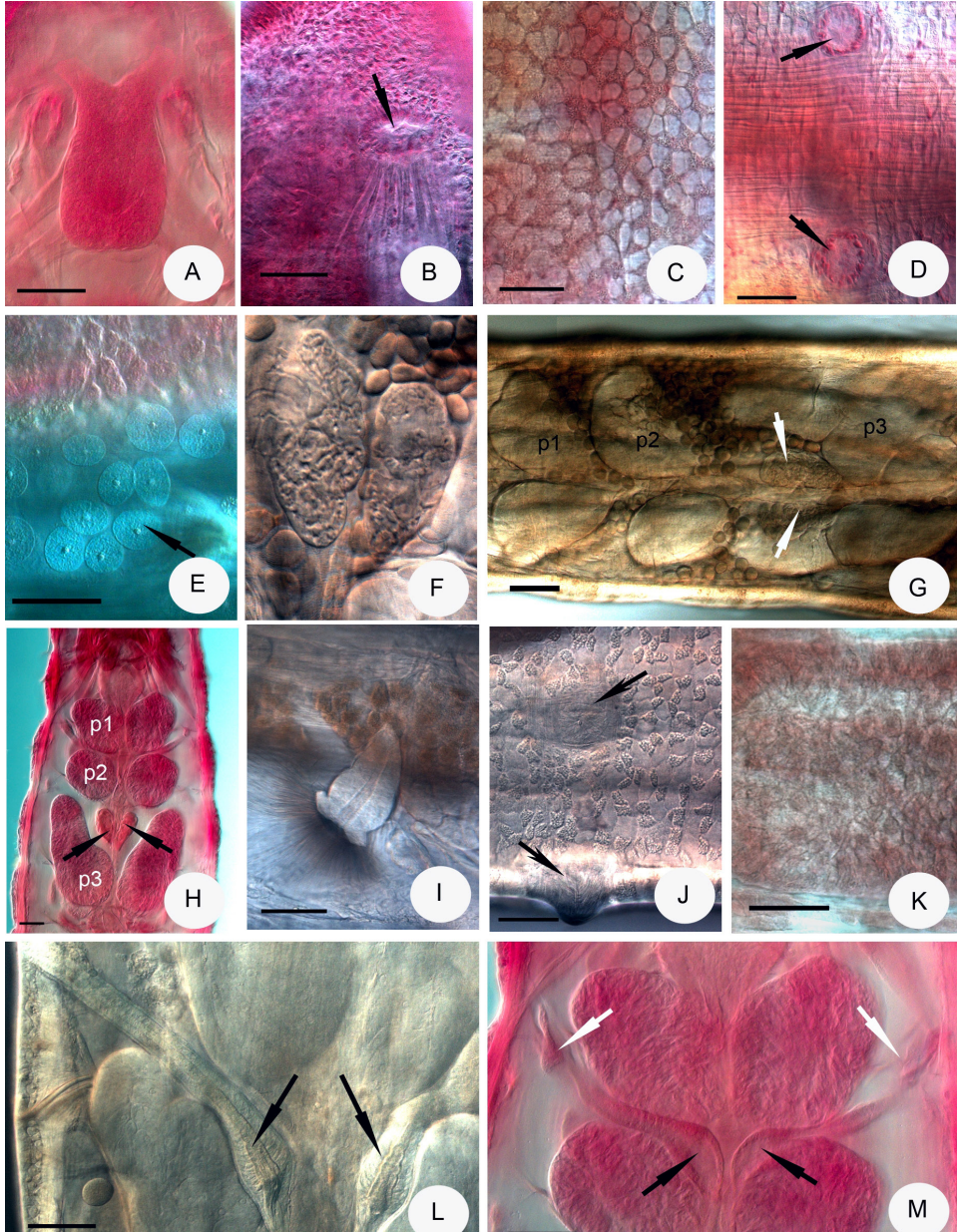
1I, K vs. Figs 3G–H); coelomocytes discoid, light brown *in vivo* (Figs 1I–J vs. Figs 3F–G) and with well visible nucleus in the fixed specimens (Fig. 1H vs. Fig. 3E); the networking cell structure of the midgut in the same place of the gut (Fig. 1M vs. Fig. 3K); shape and size of sperm funnels (Figs 2C–G vs. Fig. 3I); form of spermathecae (Figs 2H–K vs. Figs 3L–M); the shape, number and size of ectal glands (Figs 2L–M vs. Fig. 3M). Only the male copulatory organs were slightly larger *in vivo* and smaller when fixed in the Korean specimens (Table 2, Figs 1G, 2B vs. Figs 3D, J). Furthermore, they prefer to live in decaying wood, as their intestine was full with decaying wood debris, similarly as in the case of the studied European specimens. In summary, the specimens collected from Korea and Europe cannot be differentiated morphologically.

Results of DNA-based analysis

In total, 4, 6 and 6 sequences were generated from the *Bryodrilus ehlersi* specimens collected from different sites in the case of ITS, CO1 and H3, respectively. Additional sequences determined in previous studies were also used for comparison (Table 1). Unfortunately, we failed to amplify the ITS region from two specimens, which was probably due to the improper hybridization of PCR primer sequences to the extracted genomic DNA. Results of molecular studies showed that the Korean *Bryodrilus ehlersi* specimens may represent a cryptic species since based on the three studied regions, sequences acquired from these specimens formed distinct lineages on the phylogenetic trees (Figs 4 & 5).

DISCUSSION

Molecular analyses based on the mitochondrial cytochrome c oxidase subunit I gene, the nuclear histone H3 gene and the nuclear ribosomal ITS region showed unambiguously that the specimens collected in South Korea and identified morphologically as *Bryodrilus ehlersi* represent a distinct evo-

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Fig. 3. Micrograph of European *Bryodrilus ehlersi*. A = Brain. B = Head pore (marked with arrow). C = Clitellar gland cells, dorsal view. D, J = Clitellar gland cells, ventral view (penial bulbs marked with arrows). E = Coelomocytes (marked with arrow). F = Oesophageal appendages in VI. G = Three pairs of pharyngeal glands, brown, round coelomocytes in coelom and oesophageal appendages, dorsal view (marked with arrows). H = Segments III–VI, dorsal view (p1–p3 = pharyngeal glands, oesophageal appendages marked with arrows). I = Sperm funnel. J = Male copulatory organ. K = Networking cell structure in XXVI. L–M = Spermathecae, dorsal view (marked with black arrows, in M ectal glands marked with white arrows). (F–G, I–J, L *in vivo*; A–E, H, K, M fixed, stained. Scale bars: 50 μm). [A, H, M micrograph of the paratype P.21.2. of *B. glandulosus* (Dózsa-Farkas, 1990) which was synonymised with *B. ehlersi* by Cech *et al.* (2012)]
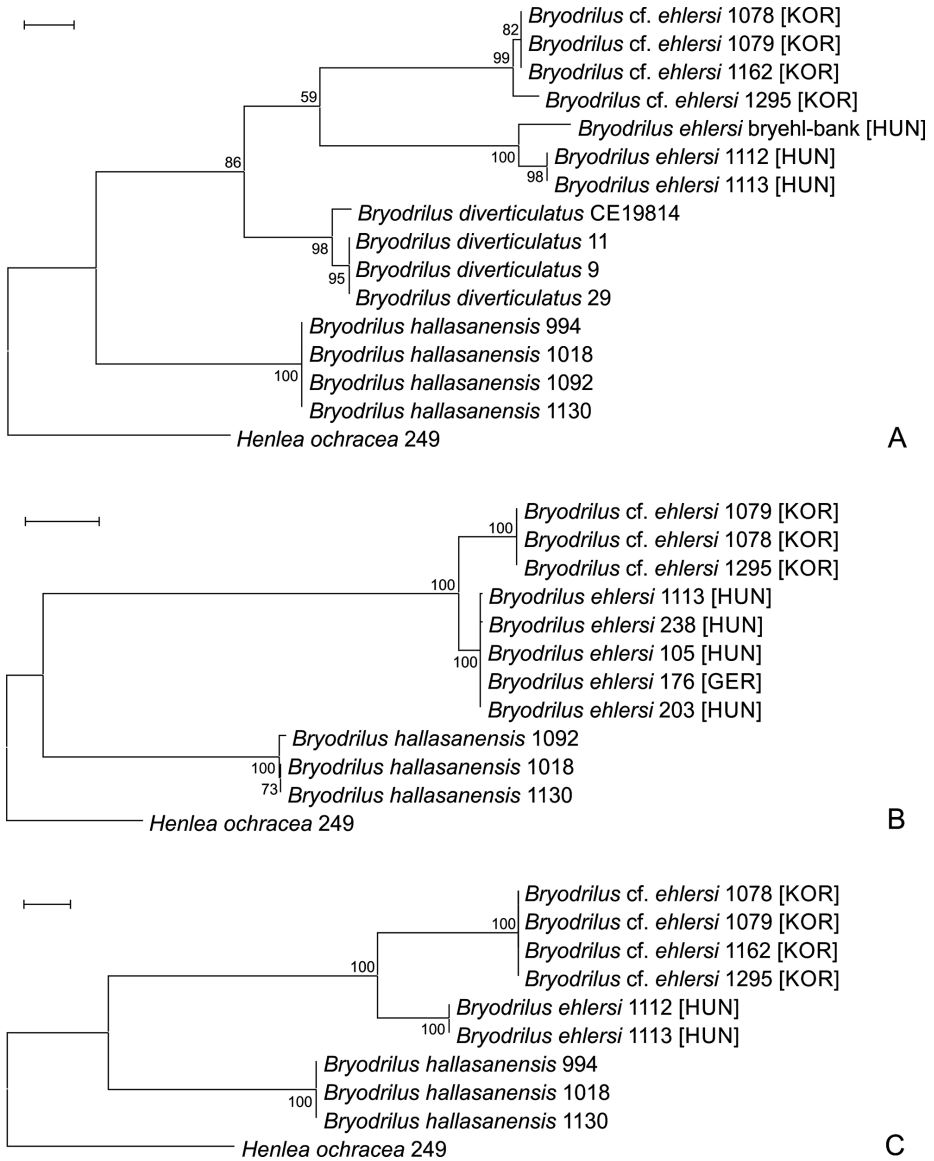
Fig. 4. *Bryodrilus* species, maximum likelihood (ML) trees of the CO1 region (A), ITS (B) and H3 genes (C). Bootstrap values greater than 50 are shown at the nodes. Accession codes of sequences with collection information are given in Table 1. A = ML tree of the CO1 gene based on 606 nucleotide positions (Tamura-Nei model). B = ML tree of the ITS region based on 765 nucleotide positions (Tamura 3-parameter model). C = ML tree of the H3 gene based on 218 nucleotide positions (Tamura 3-parameter model). Scale bars, 0.05 substitutions per nucleotide position (except for H3, where the scale is 0.01). For *B. ehlersi* specimens, the abbreviation of sample origin is shown in brackets (KOR = Korea, HUN = Hungary, GER = Germany)
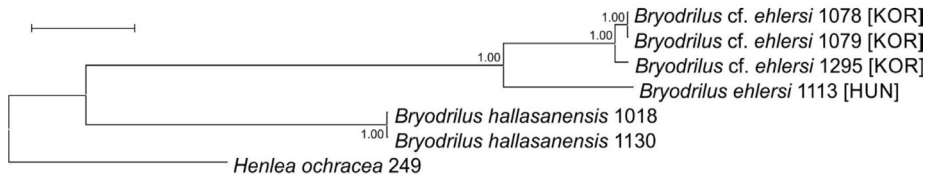
Fig. 5. *Bryodrilus* species, concatenated phylogenetic tree based on 1599 nucleotide positions (which comprised the CO1, H3 and ITS genetic markers). Bayesian posterior probabilities are shown at the nodes. Scale bar, 0.05 substitutions per nucleotide position. For *B. ehlersi* specimens, the abbreviation of sample origin is shown in brackets (KOR = Korea, HUN = Hungary)

lutionary lineage. Therefore, the Korean population may represent a cryptic species, since no unambiguous distinguishing morphological features were found in comparison with *B. ehlersi* specimens previously (see details in Cech *et al.* 2012) and recently collected from different sites in Central Europe (Hungary: Mt. Villány, Mt. Bakony, Mt. Gerecse, Kőszeg Mts and Börzsöny Mts; Germany: near Munich). Decaying wood debris and soil samples from the type locality of *Bryodrilus ehlersi* (Harz Mts, Germany) was also collected twice in July and December 2019 by W. Rackow (National Park Harz), but unfortunately not any worms belonging to this species were possible to be extracted (7 samples, data not shown). Hence, Korean specimens could not be formally described as a different species, since it was not possible to compare them with specimens collected from the type locality of *B. ehlersi*.

Bryodrilus ehlersi is common in Northern and Central Europe (Schmelz & Collado 2010). Outside Europe, this species has been recorded from Canada (Nurminen 1973*a*), the Arctic (Piper *et al.* 1982) and Lake Bajkal (Nurminen 1973*b*). The taxonomic identity of these nominal extra-European populations should also be confirmed by DNA-based methods in the future.

Revealing cryptic genetic diversity in some cases resulted in the description of new enchytraeid species. Previously, most of them were described from Europe (Westheide & Graefe 1992, De Wit & Erséus 2010, Martinsson *et al.* 2015*a,b*, Martinsson *et al.* 2018), but two cryptic species pairs were recorded from areas outside Europe (from South Africa and from Japan), resulting in the description of the new species *Grania cryptica* (Prantoni *et al.* 2016) and *Enchytraeus japonensis* (Nakamura 1993).

In this paper, it has been demonstrated that the importance of DNA-based analyses in the taxonomy of annelid species is not restricted to provide barcodes during the description of a new species for subsequent studies, but is also a tool for the recognition of cryptic diversity, which could initiate new species descriptions in the future.

<center>*</center>

REFERENCES

Ávila-Jiménez, M. L., Solhøy, T., Gwiazdowicz, D. J., Fjellberg, A., Dózsa-Farkas, K., Monson, F., DeSmet, W. H., Stur, E., Ekrem, T. & Coulson, S. J. (2019): The soil invertebrate fauna of Edgeøya, Svalbard: Arctic landscape community composition reflects biogeography patterns. – *Polar Biology* **42**: 837–850. https://doi.org/10.1007/s00300-019-02471-x

Cech, G., Boros, G. & Dózsa-Farkas, K. (2012): Revision of Bryodrilus glandulosus (Dózsa-Farkas, 1990) and Mesenchytraeus kuehnelti Dózsa-Farkas, 1991 (Oligochaeta: Enchytraeidae) using morphological and molecular data. – *Zoologischer Anzeiger* **251**: 253–262. https://doi.org/10.1016/j.jcz.2011.09.005

Colgan, D. J., McLauchlan, A. A., Wilson, G. D. F., Livingston, S. P., Edgecombe, G. D., Macaranas, J., Cassis, G. & Gray, M. R. (1998): Histone H3 and U2 snRNA DNA sequences and arthropod molecular evolution. – *Australian Journal of Zoology* **46**: 419–437. https://doi.org/10.1071/ZO98048

De Wit, P. & Erséus C. (2010): Genetic variation and phylogeny of Scandinavian species of Grania (Annelida: Clitellata: Enchytraeidae), with the discovery of a cryptic species. – *Journal of Zoological Systematics and Evolutionary Research* **48**: 285–293. https://doi.org/10.1111/j.1439-0469.2010.00571.x

Dózsa-Farkas, K., Porco, D. & Boros, G. (2012): Are Bryodrilus parvus Nurminen, 1970 and Bryodrilus librus (Nielsen and Christensen, 1959) (Annelida: Enchytraeidae) really different species? A revision based on DNA barcodes and morphological data. – *Zootaxa* **3276**: 38–50. https://doi.org/10.11646/zootaxa.3276.1.2

Dózsa-Farkas, K., Felföldi, T. & Hong, Y. (2015): New enchytraeid species (Enchytraeidae, Oligochaeta) from Korea. – *Zootaxa* **4006**(1): 171–197. https://doi.org/10.11646/zootaxa.4006.1.9

Dózsa-Farkas, K. & Felföldi, T. (2017): Comparative morphological and molecular taxonomic study of six Achaeta species (Clitellata: Enchytraeidae) with the description of a new Achaeta species from Kőszeg Mountains, Hungary. – *Zootaxa* **4273**(2): 177–194. https://doi.org/10.11646/zootaxa.4273.2.2

Dózsa-Farkas, K., Csitári, B. & Felföldi, T. (2017): A new Cernosvitoviella species (Clitellata: Enchytraeidae) and its comparison with other Cernosvitoviella species from Sphagnum mires in Hungary. – *Zootaxa* **4254**(3): 322–338. https://doi.org/10.11646/zootaxa.4254.3.2

Dózsa-Farkas, K., Felföldi, T., Nagy, H. & Hong, Y. (2018): New enchytraeid species from Mount Hallasan (Jeju Island, Korea) (Enchytraeidae, Oligochaeta). – *Zootaxa* **4496**(1): 337–381. https://doi.org/10.11646/zootaxa.4496.1.27

Dózsa-Farkas, K., Felföldi, T., Nagy, H. & Hong, Y. (2019*a*): Two new enchytraeid species from Jeju Island, Korea (Annelida, Clitellata). – *ZooKeys* **824**: 87–108. https://doi.org/10.3897/zookeys.824.25544

Dózsa-Farkas, K., Nagy, H., Felföldi, T. & Hong, Y. (2019*b*): Decimodrilus, a new enchytraeid genus from Korea (Annelida, Clitellata, Enchytraeidae). – *Zootaxa* **4661**: 385–399. https://doi.org/10.11646/zootaxa.4661.2.7

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994): DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. – *Molecular Marine Biology and Biotechnology* **3**: 294–299.

Huelsenbeck, J. P. & Ronquist, F. (2001): MRBAYES: Bayesian inference of phylogeny. – *Bioinformatics* **17**: 754–755. https://doi.org/10.1093/bioinformatics/17.8.754

Kane, R. A. & Rollinson, D. (1994): Repetitive sequences in the ribosomal DNA internal transcribed spacer of Schistosoma haematobium, Schistosoma intercalatum and Schistosoma mattheii. – *Molecular and Biochemical Parasitology* **63**: 153–156. https://doi.org/10.1016/0166-6851(94)90018-3

Kumar, S., Stecher, G. & Tamura, K. (2016): MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. – *Molecular Biology and Evolution* **33**: 1870–1874. https://doi.org/10.1093/molbev/msw054

Martinsson, S., Rota, E. & Erséus, C. (2015*a*): Revision of Cognettia (Clitellata, Enchytraeidae): reestablishment of Chamaedrilus and description of cryptic species in the sphagnetorum complex. – *Systematics and Biodiversity* **13**(3): 257–277. https://doi.org/10.1080/14772000.2014.986555

Martinsson, S., Rota, E. & Erséus, C. (2015*b*): On the identity of Chamaedrilus glandulosus (Michaelsen, 1888) (Clitellata, Enchytraeidae), with the description of a new species. – *ZooKeys* **501**: 1–14. https://doi.org/10.3897/zookeys.501.9279

Martinsson, S., Dózsa-Farkas, K., Rota, E. & Erséus, C. (2017): Placing the forgotten: On the phylogenetic positions of Euenchytraeus and Chamaedrilus in an updated enchytraeid phylogeny (Clitellata: Enchytraeidae). – *Invertebrate Systematics* **31**(1): 85–90. https://doi.org/10.1071/IS16042

Martinsson, S. & Erséus, C. (2018): Cryptic diversity in supposedly species-poor genera of Enchytraeidae (Annelida: Clitellata). – *Zoological Journal of the Linnean Society* **183**: 749–762. https://doi.org/10.1093/zoolinnean/zlx084

Matamoros, L., Rota, E. & Erséus, C. (2012): Cryptic diversity among the achaetous *Marionina* (Annelida, Clitellata, Enchytraeidae). – *Systematics and Biodiversity* **10**(4): 509–525. https://doi.org/10.1080/14772000.2012.723640

Nakamura, Y. (1993): A new fragmenting enchytraeid species, *Enchytraeus japonensis* from a cropped Kuroboku soil in Fukushima, Northern Japan (enchytraeids in Japan 5). – *Edaphologia* **50**: 37–39.

Nielsen, C. O. & Christensen, B. (1959): The Enchytraeidae – critical revision and taxonomy of European species (Studies on Enchytraeidae VII). – *Natura Jutlandica* **8–9**: 1–160.

Nurminen, M. (1970): Records of Enchytraeidae (Oligochaeta) from the west coast of Greenland. – *Annales Zoologici Fennici* **7**: 199–209.

Nurminen, M. (1973*a*): Enchytraeidae (Oligochaeta) from the Arctic archipelago of Canada. – *Annales Zoologici Fennici* **10**: 404–411.

NURMINEN, M. (1973*b*): Enchytraeidae (Oligochaeta) from the vicinity of the Lake Baikal, Siberia. – *Annales Zoologici Fennici* **10**: 478–482.

O'CONNOR, F. B. (1962): The extraction of Enchytraeidae from soil. Pp. 279–285. – *In*: MURPHY, P. W. (ed.): *Progress in soil zoology*. – Butterworths Publishers, London.

PIPER, S. R., MACLEAN, S. F. & CHRISTENSEN, B. (1982): Enchytraeidae (Oligochaeta) from taiga and tundra habitats of northeastern U.S.S.R. – *Canadian Journal of Zoology* **60**(11): 2594–2609. https://doi.org/10.1139/z82-334

PRANTONI, A. L., DE WIT, P. & ERSÉUS, C. (2016): First reports of Grania (Clitellata: Enchytraeidae) from Africa and South America: molecular phylogeny and descriptions of nine new species. – *Zoological Journal of the Linnean Society* **176**: 485–510. https://doi.org/10.1111/zoj.12333

SCHMELZ, R. M. & COLLADO, R. (2010): A guide to European terrestrial and freshwater species of Enchytraeidae (Oligochaeta). – *Soil Organisms* **82**(1): 1–176.

SCHMELZ, R. M., BEYLICH, A., BOROS, G., DÓZSA-FARKAS, K., GRAEFE, U., HONG, Y., RÖMBKE, J., SCHLAGHAMERSKY, J. & MARTINSSON, S. (2017): How to deal with cryptic species in Enchytraeidae, with recommendations on taxonomical descriptions. – *Opuscula Zoologica (Budapest)* **48**(Suppl. 2): 45–51. https://doi.org/10.18348/opzool.2017.S2.45

UDE, H. (1892): Ein neues Enchytraeiden-Genus. – *Zoologischer Anzeiger* **15**: 344–345.

VAIDYA, G., LOHMAN, D.J. & MEIER, R. (2011): SequenceMatrix: concatenation software for the fast assembly of multigene datasets with character set and codon information. *Cladistics* **27**: 171–180. https://doi.org/10.1111/j.1096-0031.2010.00329.x

WESTHEIDE, W. & GRAEFE, U. (1992): Two new terrestrial Enchytraeus species (Oligochaeta, Annelida). – *Journal of Natural History* **26**: 479–488.