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Enchytraeus demutatus sp. nov. (Enchytraeidae, Oligochaeta) has characters hitherto unrecorded in the genus

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Abstract

A new species of enchytraeids with peculiar characters is described, *Enchytraeus demutatus* sp. nov. The species differs from all known species of *Enchytraeus* in features of the oesophageal appendages and the male efferent apparati. The oesophageal appendages insert ventro-laterally below the pharyngeal pad and not dorsally behind the pad, as is usual in the genus; furthermore, their histology is different. The male efferent apparati have each an unusually wide and short vas deferens, and accessory glands are attached to the inner surface of the male glandular bulb, in close vicinity to the male pore. The remaining characters agree with the genus diagnosis of *Enchytraeus*, notably the presence of testis sacs and oesophageal appendages. The bisetose worms are about 5 mm long and 0.2–0.3 mm wide. The peculiarities of the oesophageal appendages raised the possibility to place the species in a new genus. DNA sequencing was performed to provide molecular identifiers and to assess the generic placement of the species. The molecular phylogenetic analysis, based on a concatenation of the six genetic markers 12S, 16S, 18S, 28S, COI and H3 placed *E. demutatus* inside the well-supported (PP = 0.99) *Enchytraeus* clade and not outside, hence the new species is to be considered a member of *Enchytraeus*. The tree includes species that cover a wide range of the morphological diversity in *Enchytraeus*; here *E. demutatus* sp. nov. groups with a sub-branch that includes the other bisetose species, also the fragmenting ones, but also one species with 3 chaetae per bundle; whereas species of the *E. buchholzi*-group and the *E. albidus*-group, respectively, form clades of their own. *E. demutatus* sp. nov. was found in soil samples derived from a tomato field of a private greenhouse farm in the regional division of Ilia, West Greece, together with abundant *E. bigeminus*.

Keywords Clitellata | soil fauna | soil biodiversity | taxonomy

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1. Introduction

A new species of enchytraeids was detected in a soil sample that two of us (C.C, P.A.) had brought to the taxonomic workshop held at the 13th International Symposium on Enchytraeidae, Versailles, June 2018. The soil sample was from a tomato field of a private greenhouse farm in Greece, and it contained two species, abundant *Enchytraeus bigeminus* Nielsen & Christensen, 1963 *sensu lato* (see Collado et al. 2012) and the new species, which is described in this paper. The species exhibited a mosaic of characters that made a genus assignment difficult: Presence of oesophageal appendages and testis sacs favoured an inclusion in *Enchytraeus*, the only enchytraeid genus where these two characters occur together. On the other hand, several details of these appendages and also details of the male efferent apparatus, to be described below, differed markedly from the known pattern in *Enchytraeus* and suggested the erection of a new genus. To clarify the position of this new species, DNA sequencing and an estimation of phylogenetic relationships was performed.

2. Material and methods

Material. The soil sample was from an experimental laboratory culture of Enchytraeidae at the Agricultural University of Athens, Greece. The soil used in the cultivation experiment stemmed from a tomato field of a private greenhouse farm in the regional division of Ilia, West Greece. Here many greenhouse crops are cultivated because of the favorably mild and humid climate, which promotes early ripening. The soil was organically enriched with compost material, but further data are not available. The exact location of the farm is unknown as well. Therefore, the regional division of Ilia is declared as type locality of the new species here.

Morphology. The first specimens of the new species were investigated *in vivo* at the 13th International Symposium on Enchytraeidae, Versailles, June 2018. These specimens were not preserved. The soil sample was transferred to the laboratory of the first author and enchytraeids were fed crunched oat flakes. After six weeks two further adult specimens could be isolated among abundant *E. bigeminus*. One of these two specimens is the holotype, the other one is paratype Nº ZMB 0002. They were investigated *in vivo*, anaesthetized by adding drops of 10 % ethanol to the water until relaxation, then cut in two halves. The respective anterior end was fixed in hot Bouin's fluid; the posterior end was fixed in 70 % ethanol and preserved in 100 % ethanol. One posterior end was subsequently sent to the laboratory of the second author to obtain DNA sequences. The anterior ends were stained with Paracarmine and whole-mounted in Canada Balsam between two coverslips. Light-microscopical investigation with interference (Nomarski) optics included a drawing tube for line drawings and a camera for photographs.

The mixed culture was maintained and fed, and parts were transferred to Agar Agar substrate. After several months more adults of the new species and cocoons appeared, and twelve further specimens were separated and prepared in the same way as the two above-mentioned anterior body ends. These 14 specimens are the type series. A culture of the new species is presently maintained by the first author at the University of A Coruña. The description, mainly based on the type series, was complemented by observations on living specimens derived from this culture.

Terms of description follow Schmelz & Collado (2010); paired structures of the reproductive organs are often referred to in the singular form.

Type material is deposited at the National History Museum Berlin (Museum für Naturkunde Berlin, collection 'Vermes', Generalkatalog Free-living worms, ZMB = previously Zoological Museum Berlin) and at the laboratory of Agricultural Zoology and Entomology of the Agricultural University of Athens in Greece.

DNA-sequencing and phylogenetic inference. DNA was extracted from the amputated posterior end of one *E. demutatus* sp. nov., using QuickExtract DNA Extraction Solution 1.0 (Epicentre, Madison, WI, USA). From the extract six genetic markers (12S, 16S, 18S, 28S, COI and H3) were amplified using PCR, following primers and programs found in Martinsson et al. (2017: Table S2). Sequencing was carried out by MWG Eurofins Operon (Edersberg, Germany), sequences were assembled in Genious 6.1.8 (https://www.geneious.com, Kearse et al. 2012), and deposited in Genbank (Table 1). For comparison, posterior ends of the following species were processed in exactly the same way (one specimen per species): *E. dichaetus* Schmelz & Collado, 2010, collected in grassland soil, Revelneset, Mo i Rana, Norway, N66.3153º, E14.1706º, 8 Sep 2014 by Christer Erséus & Endre Willassen, and *E. doerjesi* Westheide & Graefe, 1992, taken from a lab culture, ECT Oekotoxikologie GmbH, Flörsheim, Germany, Dec 2006 by Jörg Römbke & Andreas Haller.

A concatenated phylogenetic analysis was run in MrBayes v3.2.6 (Ronquist et al. 2012) using all 6 genetic markers (aligned using MAFFT; Katoh et al. 2002), from 21 taxa combined into a 3964 characters long dataset.

The taxa were mainly previously published *Enchytraeus* species and outgroups selected from the enchytraeid genera considered most closely related to *Enchytraeus* (Table 1), as suggested by the results of Martinsson et al. (2017). The dataset was partitioned according to genetic marker, with COI and H3 further partitioned according to codon position. The specimens CE5408 *Enchytraeus albellus* Klinth, Erséus & Rota, 2019 (Erséus et al. 2019) and CE803 *Fridericia magna* Friend, 1899 were heterozygous in H3, but we only included one randomly selected allele from each specimen in the analysis. The partitions were unlinked, allowing for different proportions of invariable sites, base frequencies, shape of gamma distribution and substitution rates, with evolutionary model estimated simultaneously with tree construction, using reversible model jump. The MCMC was set to run for 100 million generations sampling every 100 000 generations, and a consensus tree was

Table 1. Species of *Enchytraeus*, specimens, country of origin, and Genbank accession numbers for the six genetic markers used in the concatenated phylogenetic analysis. Newly generated sequences in boldface, other sequences from the following sources: [1] Erséus et al. 2010, [2] Martinsson et al. 2017, [3] Erséus et al. 2019. * This specimen was misidentified in Erséus et al. 2010 as *E. luxuriousus* Schmelz & Collado, 1999.

Species	Country	ID	12S	16S	18S	28S	COI	H3
Enchytraeus demutatus sp. nov.	Greece	CE35377	**MN248674**	**MN248682**	**MN248683**	**MN248688**	**MN248695**	**MN248697**
E. albellus Klinth, Erséus & Rota, 2019	Norway	CE5408	**MN248673**	**MN248680**	**MN248686**	**MN248691**	MK266875[3]	MK266927[3]
E. albidus Henle, 1837	Sweden	CE521	GU901693[1]	GU901785[1]	GU901870[1]	GU901956[1]	GU902047[1]	MK266911[3]
E. bigeminus s. lat. Nielsen & Christensen, 1963*	Germany (lab culture)	CE2175	GU901700[1]	GU901792[1]	GU901877[1]	GU901963[1]	GU902053[1]	**MN248698**
E. buchholzi Vejdovsky, 1878	Russia	CE724	GU901694[1]	GU901786[1]	GU901871[1]	GU901957[1]	GU902048[1]	KX644876[2]
E. bulbosus Nielsen & Christensen, 1963	Sweden	CE798	GU901695[1]	GU901787[1]	GU901872[1]	GU901958[1]	GU902049[1]	MK266943[3]
E. christenseni Dózsa-Farkas, 1992	Sweden	CE805	GU901696[1]	GU901788[1]	GU901873[1]	GU901959[1]	GU902050[1]	MK266945[3]
E. coronatus Nielsen & Christensen, 1959	Sweden	CE6179	KX618740[2]	KX618760[2]	KX618768[2]	KX618794[2]	KX618734[2]	KX644877[2]
E. crypticus Westheide & Graefe, 1992	Germany (lab culture)	CE2183	GU901697[1]	GU901789[1]	GU901874[1]	GU901960[1]	GU902055[1]	MK266942[3]
E. dichaetus Schmelz & Collado, 2010	Norway	CE24345	**MN248676**	**MN248678**	**MN248684**	**MN248692**	**MN248694**	**MN248700**
E. doerjesi Westheide & Graefe, 1992	Germany (lab culture)	CE2187	**MN248671**	**MN248677**	–	MN248689	**MN248696**	**MN248699**
E. japonensis Nakamura, 1993	Japan	CE881	GU901698[1]	GU901790[1]	GU901875[1]	GU901961[1]	GU902051[1]	**MN248701**
E. lacteus Nielsen & Christensen, 1961	Sweden	CE813	GU901699[1]	GU901791[1]	GU901876[1]	GU901962[1]	GU902052[1]	MK266946[3]
E. moebii (Michaelsen, 1885)	Sweden	CE965	**MN248672**	**MN248679**	**MN248685**	**MN248690**	MK266806[3]	MK266905[3]
E. norvegicus Abrahamsen, 1968	Sweden	CE804	GU901701[1]	GU901793[1]	GU901878[1]	GU901964[1]	MK266893[3]	MK266944[3]
E. polatdemiri Arslan & Timm, 2018	Turkey	CE14151	**MN248675**	**MN248681**	**MN248687**	**MN248693**	MK266890[3]	MK266939[3]
Enchytronia parva Nielsen & Christensen, 1959	Sweden	CE806	GU901702[1]	GU901794[1]	GU901879[1]	GU901965[1]	GU902056[1]	**MN248703**
Fridericia magna Friend, 1899	Sweden	CE803	GU901712[1]	GU901804[1]	GU901889[1]	GU901975[1]	GU902066[1]	**MN248702**
Marionina clavata Nielsen & Christensen, 1961	Sweden	CE849	GU901746[1]	GU901837[1]	GU901921[1]	GU902009[1]	GU902097[1]	**MN248704**
Marionina spicula (Leuckart, 1847)	Sweden	CE2561	KX618755[2]	KX618763[2]	KX618769[2]	KX618802[2]	KX618730[2]	KX644887[2]
Stephensoniella sterreri (Lasserre & Erséus, 1976)	USA	CE941	GU901762[1]	GU901851[1]	GU901934[1]	GU902026[1]	GU902111[1]	KX644888[2]

summarized after discarding the initial 25% as burnin. Tracer v1.5 (Rambaut et al. 2018) was used to confirm that the analysis had converged. The tree was edited in GIMP 2.8.10.

Abbreviations used in text and figures

viv – observations based on living specimens, **fix** – observations based on preserved material, **V**, **VI** – 5th segment, 6th segment (etc.), **5/6** – septum between 5th and 6th segment (etc.), **af** – afferent fascicle of pharyngeal gland, **am** – spermathecal ampulla, **bc** – border cells of clitellum, **br** – brain, **bs** – bursal slit, **cg** – chloragocytes, **ch** – chaetae, **eg** – spermathecal ectal gland, **en** – spermathecal ental duct, **gl** – accessory gland, **mb** – male ('penial') bulb, **mp** – male pore, **ne** – nephridium, **oa** – oesophageal appendage, **oe** – oesophagus, **pg** – pharyngeal gland, **pp** – pharyngeal pad, **se** – septum, **st** – spermatheca, **te** – testis sac, **tf** – thickened floor of oesophagus, **tl** – thickened lip on male bursa, **vn** – ventral nerve cord.

3. Results

Enchytraeus demutatus sp. nov.

(Figures 1, 2)

Type material. Holotype. ZMB 12202, anterior body end, stained whole mount. Regional division of Ilia, Greece, 37°18'N–38°06'N, 21°54'–22°12'E. Organically enriched soil from tomato greenhouse plantation, soil sample taken by Chr. Chalkia. Specimen isolated from soil sample and fixed 13 Aug 2018 by R.M. Schmelz at the University of A Coruña.

Paratypes. ZMB 12203, same data as holotype. ZMB 12204-12211, stained whole mounts. Specimens reared in Agar Agar substrate, isolated and fixed Dec 2018. Four paratype specimens in the collection of the laboratory of Agricultural Zoology and Entomology of the Agricultural University of Athens in Greece, without accession numbers.

Further material. C. 20 specimens, adult, subadult, and juvenile, from cultures in soil or Agar Agar, investigated *in vivo*, not preserved.

DNA sequences. Holotype: Anterior part: ZMB 12202. Posterior part, processed for DNA extraction: CE35377 (C. Erséus Collection, see Table 1, Fig. 3).

Etymology. The Latin adjective *demutatus* means changed, altered, transformed (perfect passive participle of *demutare*). Named for the characters that differ from the patterns hitherto known in the genus.

Description

Body length c. 5 mm (viv), 4.2–6.2 mm (fix, N = 13), diameter 0.14–0.2 mm at V, 0.2–0.37 mm at XII (fix, N = 14). **Segment number** 25–29, mostly 27, 28 (10 out of 14 specimens). **Chaetae** two per bundle, ectally straight, entally curved, laterals and ventrals alike, c. 35–42 µm long, 2.5–3 µm wide, largest in some anterior and posterior segments (e.g., V–VIII, XXII–XXIV). Occasionally single bundles with 3 chaetae.

Prostomium rounded, about as long as wide (viv) or shorter (fix), head pore at 0/I or at the beginning of I, a longitudinal slit, inconspicuous. **Epidermal gland cells** often absent or indistinguishable from rest of epidermis with the methods used (viv, fix), occasionally seen (viv) as small scattered cells in anterior 8 segments, slightly darker than rest of epidermis. Body wall thin, mostly 4–6 µm, thicker in anterior segments (8–12 µm). **Septa** thin, first septum at 4/5. **Brain** (Fig. 1A) 2–2.5x as long as wide (c. 120 µm by 60 µm, viv), posteriorly rounded or truncated, sides parallel, anteriorly convex, here with agglomerations of perikarya. **Ventral nerve cord** (Fig. 1B) medullar, distinct segmental swellings in II, III and IV; from IV on swellings less conspicuous. **Post-pharyngeal bulbs** (not shown in Fig. 1) two pairs of spherical bodies, one inner contiguous pair mid-dorsally behind pharyngeal pad, one outer pair laterally within afferent fascicles of pharyngeal glands. **Pharyngeal pad** (Fig. 1A) oval, in II and III. **Pharyngeal glands** (Figs 1A, 2A) three pairs in IV, V, VI, all alike, the pair in VI sometimes reduced in size; with dorsal and primary ventral lobes of about equal size, no secondary lobes; dorsal lobes adjacent mid-dorsally, separate or united. Afferent fascicles in IV (Fig. 1A) with an additional oval swelling between ventral lobes and pharyngeal ganglion (fix). **Oesophageal appendages** (Fig. 1A,B) paired, in III–V; elongate, unbranched, blind-ending, sausage-like tubes with smooth outer surface and about equal diameter throughout, opening latero-ventrally into pharynx at 2/3, below pharyngeal pad; tubes with interstitial tissue and irregular, interconnected lacunae, probably a winding canal, not ciliated, lacunae often collapsed, especially in distal two thirds (towards blind end), then aspect spongy (viv) or solid (fix). Length of tubes c. 340–400 µm, diameter 12–20 µm, often narrowest towards blind end and widest in middle of proximal third. Pharyngeal/oesophageal floor thickened ventrally and ventro-laterally immediately behind insertion points of oesophageal appendages. **Intestinal diverticula** absent. **Intestine** widening in XIII, behind origin of dorsal blood vessel. **Pars tumida of midgut** in XIX–XXI, one segment length, mostly in XX (9 out of 13 specimens), cells c. 20–25 µm high, non-staining (fix), covering entire ventral half of gut cross-section. **Dorsal blood vessel** from XIII, anterior bifurcation in prostomium, circum-oesophageal connectives uniting to ventral vessel in IV; four pairs of lateral commissurals present: two pairs at 3/4, connecting ventrally with connectives, one pair in IV anterior to septum 4/5 and one pair in V anterior to septum 5/6, connecting with ventral vessel. **Nephridia** (Fig. 1A) six preclitellar pairs, at 4/5 to 9/10, first postclitellar pair at 13/14. Anteseptale with funnel only, postseptale oval, c. 1.2–1.5x as long as high, short efferent duct rising postero-dorsally, nephridioporus with terminal vesicle. **Coelomocytes** (Fig. 2D) not numerous, without refractile vesicles, often partly hyaline, length 15–25 µm, variable in shape and texture, largest cells pear-shaped and filled with pale vesicles.

Gonadal segments in the usual position: testes and sperm funnels in XI, ovaries and vasa deferentia in XII, female pores at 12/13. **Clitellum** (Figs 1D, 2B,E) in XII–

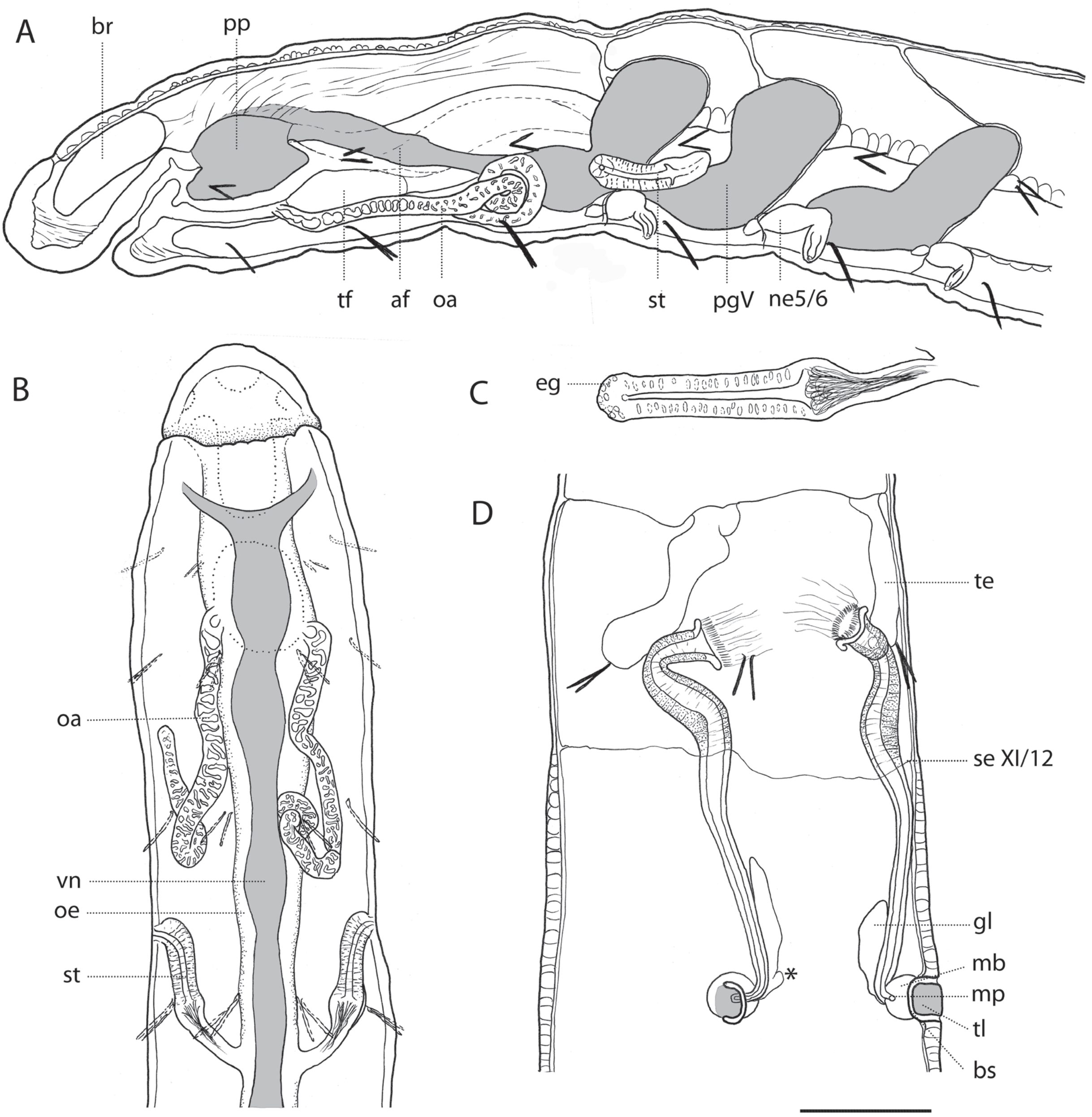

Figure 1. *Enchytraeus demutatus* sp. nov. (**A**) Anterior body end, lateral view, paratype ZMB 12203. Shaded-grey: pharyngeal pad, afferent fascicles and pharyngeal glands, a functional unit. (**B**) Anterior body end, ventral view, holotype, pharyngeal glands omitted. Shaded grey: ventral nerve cord. Brain and pharyngeal pad indicated by dotted lines. (**C**) Spermatheca, as seen in living specimen (comp. Fig. 2C). (**D**) Male reproductive organs in XI, XII, paratype ZMB 12205. Shaded grey: thickened lip on male bursa, surrounded by bursal slit. Asterisk: spherical accessory gland, attached to elongated gland. A, B, D from stained whole mounts, C from live photograph. Scale bars = 100 µm (same bar for A, B, D).

XIII, two segments, girdle-shaped, flat (height 10–12 µm), hyalocytes and granulocytes alternating, arranged in indefinite rows or in reticulate pattern, alike on all sides, except mid-ventrally between bursal slits, here slightly fewer hyalocytes than in the rest; diameters granulocytes c. 8 µm, hyalocytes c. 10 µm (fix). Aspects in living specimens: granulocytes as dots equidistant from each other (Fig. 2B). **Testis sacs** (Fig. 1C) present, continuous with testes, paired, small, drop-shaped. No seminal vesicle, no developing sperm in coelom, mature spermatozoa on top of sperm funnel. **Sperm funnel** (Fig. 1D) elongate, c. 110 µm, widest at collar (42 µm); glandular funnel body

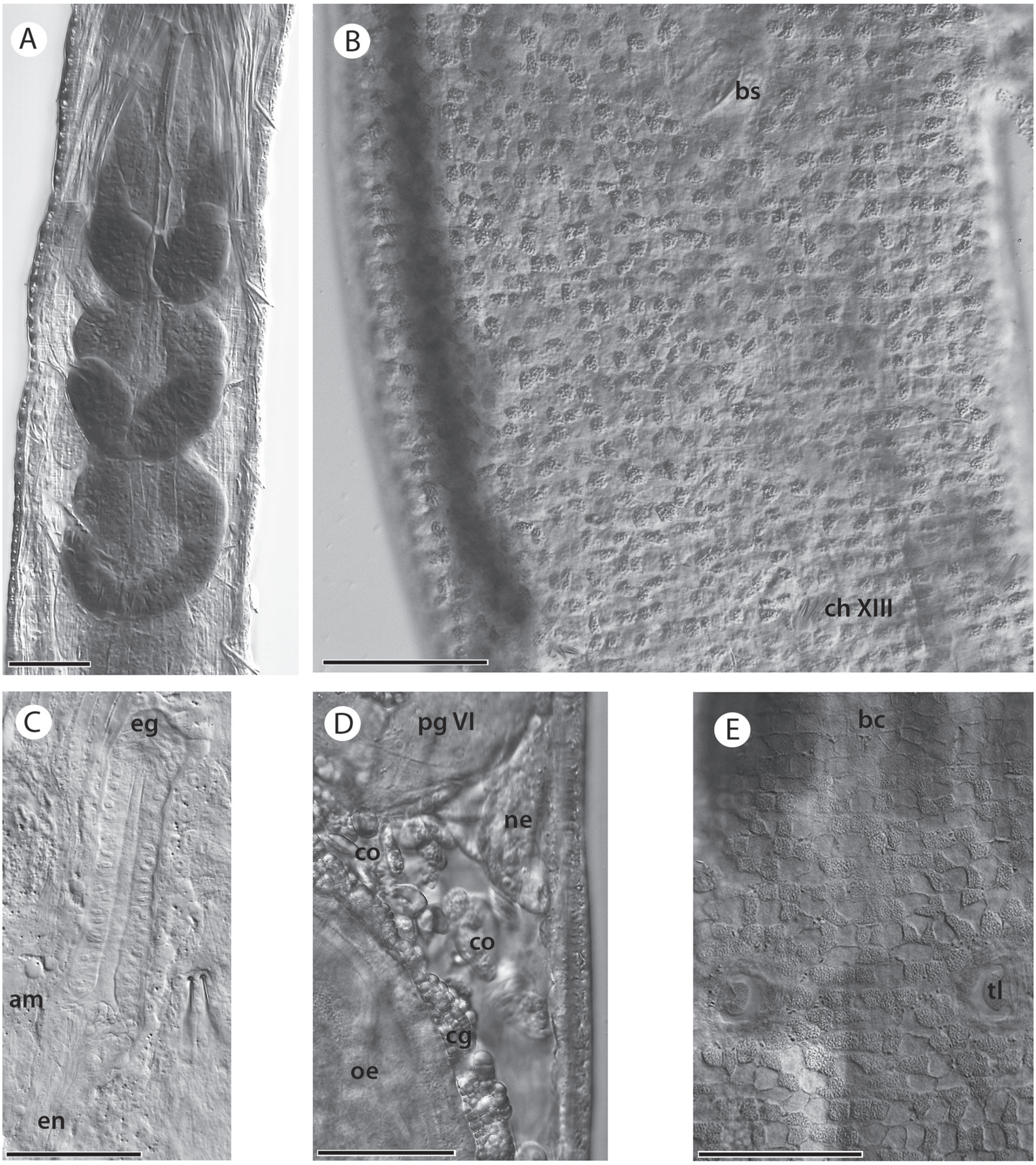

Figure 2. *Enchytraeus demutatus* sp. nov. (**A**) Pharyngeal glands and further details in III–VI, dorsal view. (**B**) Clitellum, ventrolateral view, specimen flattened. (**C**) Spermatheca. The dots in the ectal duct wall and the ectal gland are cell nuclei. (**D**) Coelomocytes. (**E**) Anterior clitellum, midventral part with bursal slits. Photographs A, E from paratype specimens, stained whole mounts, B–D from living specimens, not preserved; in all, anterior body end to the top. Scale bars 100 µm in A, B, E, 50 µm in C, D.

with varying thickness: narrowing beneath collar from 24–28 µm to 14–18 µm, then widening again to about 30–34 µm, then slightly narrowing towards 11/12, gradually merging into vas deferens (24 µm); canal slightly asymmetrical. Vas deferens (Fig. 1D) short, straight, not coiled, length 200 µm (fix), c. twice as long as sperm funnel, entally almost as wide as sperm funnel (c. 20 µm), canal 15 µm wide; vas gradually narrowing towards male pore, ectal diameter 10 µm. **Male pores** (Fig. 1D) on ental roof of eversible bursa, surrounded by a small and densely nucleated ('penial') bulb, bursal slit (Figs 1D, 2B,E) C-shaped, the 'C' enclosing a thickened lip. One elongate and 0–3 small spherical, densely nucleated **accessory glands** attached to ectal part of vas deferens (Fig. 1D). Elongate glands c. 60–70 µm long, 3x as long as wide, attached to **male bulb** adjacent to male pore, canal not observed. Subneural glands and other accessory glands absent. **Spermathecae** (Figs 1A,C, 2C) with minute ectal gland, moderately long ectal duct, ampulla without diverticula, and ental duct; total length c. 160 µm (viv, fix, not contracted). Ectal glands near ectal pore, minute, sessile, as an anterior swelling of ectal duct, not seen in all specimens. Ectal duct stout, contractile, not glandular, c. 2.5x as long as wide (e.g., 92 µm by 24 µm, viv, 60 µm by 18 µm, fix), outer surface smooth, not wavy or undulating; canal lined with cuticle, gradually widened from 3 µm ectally to 8 µm entally (viv), cuticle with funnel-shaped widening at base of ampulla. Ampulla slightly wider than ectal duct (c. 35 µm), without diverticula, narrowed into an ental duct two thirds as long as ectal duct (c. 60 µm). Sperm in ampulla and ental duct, sperm heads arrested in ampullar epithelium, tails extending freely into ental duct. Separate attachments of spermathecae to lateral sides of oesophagus.

4. Remarks

Morphology, generic placement. Presence of testis sacs (as opposed to seminal vesicles, see Schmelz & Collado 2010: 29) and paired tubular oesophageal appendages agree with the genus diagnosis of *Enchytraeus*, the only genus of Enchytraeidae where these two structures occur together. Further characters that in combination support the placement of this species in *Enchytraeus* are as follows: head pore at 0/1, chaetae straight distally, brain not deeply incised posteriorly, pharyngeal glands without secondary lobes, nephridial anteseptale with funnel only, no intestinal diverticula, dorsal blood vessel not anterior to clitellum, coelomocytes one type only, male pores in eversible bursa with glandular ('penial') bulb, spermathecae attached to oesophagus.

Several details of the oesophageal appendages, however, differ from the pattern hitherto known in the genus: (1) In *E. demutatus*, the attachments are separate, ventrolateral, and below the pharyngeal pad at 2/3, which contrasts with a – so far genus-diagnostic – adjacent or joint dorsal attachment of these appendages behind the pharyngeal pad in III. The connection with the pharyngeal walls is so far unique in the family: in all other species of enchytraeids with oesophageal appendages the connection is further back, with the walls of the oesophagus: *E. demutatus* has, as it were, pharyngeal and not oesophageal appendages. (2) The lumen of the appendages is organized as a series of interconnected lacunae, which probably form a canal that is much winded inside the appendage and surrounded by interstitial tissue; when collapsed the appearance is spongy. In other species of *Enchytraeus* the canal may be winded as well but then the walls follow the canal windings so that the whole tube is winded, and not only the canal inside the tube. The pattern in *E. demutatus* is similar to the one in the oesophageal appendages of *Hemienchytraeus*, where, however, the appendages are connected with the oesophagus dorsally in III, behind the pharyngeal pad.

Further peculiarities that deviate from the patterns known in *Enchytraeus* are found in the male efferent apparatus. (1) Sperm funnel and vas deferens are not marked off from each other; there is no abrupt change in diameter at 11/12, as is usual in the genus and most other species of Enchytraeidae. Actually, the two parts are distinguishable only by the glandular nature of the funnel walls, i.e., the anterior part of the organ in XI. (2) The vas deferens is short to extremes, that is, it covers the distance from 11/12 to the male pores in a straight line without any windings or coils. It is also wider than in many species of the genus, even though widenings of the vas deferens have been reported for species of the *E. albidus* group (see Erséus et al. 2019), where, on the other hand, the vas is much longer. The overall shape of the male efferent apparatus is similar to conditions in some species of *Cernosvitoviella*, a genus of aquatic or semi-aquatic species with curved and nodulated chaetae, incised brain, secondary pharyngeal glands, and entally free and blind-ending spermathecae. (3) Accessory bodies or glands attached to the male bulb are also new for the genus. As apparent outgrowths of the male bulb or other epithelia very close to the male pore, these structures are different from the accessory glands in species of the *E. albidus* group, where the glands surround the male pore at some distance, and separately from the central male bulb (comp. Erséus et al. 2019).

In enchytraeids oesophageal appendages are a 'strong' character at the genus level: presence/absence, overall shape, histology, location, and especially attachment points are diagnostic of genera (see the compilation in Schmelz & Collado 2010: 22f.) and they are usually the same in all species of a genus – with the notable exceptions of *Oconnorella* (see Chen et al. 2006), *Marionina* (see Xie & Rota 2001), and *Hemifridericia* (see Dózsa-Farkas & Felföldi 2015) – or they vary within clearly defined limits. Therefore the idiosyncratic pattern of shape, histology and attachment point, together with the peculiarities of the male efferent apparatus, raised the possibility that the new species would be better allocated to a new genus, perhaps as a sister group of a monophyletic *Enchytraeus* (Erséus et al. 2010). The alternative *Enchytraeus*/new genus was decided by DNA sequencing.

Molecular phylogenetic analysis. The molecular phylogenetic analysis, based on a concatenation of six genetic markers, placed *E. demutatus* inside the well-supported (PP = 0.99) *Enchytraeus* clade and not outside, hence the new species is to be considered a member of *Enchytraeus*. The tree, which includes species that cover a wide range of the morphological diversity in *Enchytraeus*, is subdivided into two main branches, one not so well-supported (PP = 0.83) clade which contains all the bisetose species included in the analysis (*E. dichaetus*, *E. japonensis* Nakamura, 1993, *E. bigeminus* Nielsen & Christensen, 1963, *E. demutatus*) and all the fragmenting species (*E. japonensis*, *E. bigeminus*). However, this branch also includes one species with 3 chaetae per bundle, *E. doerjesi*. [Side note: The specimen representing *E. bigeminus* in this tree is identical with the one identified as *E. luxuriosus* Schmelz & Collado, 2000 in Erséus et al. (2010), the latter being a misidentification.] The other branch, which is well-supported, (PP = 1) contains two subbranches, one with species of the marine littoral *E. albidus* group (see Erséus et al. 2019) and another one with species of the *E. buchholzi* group as circumscribed in Schmelz & Collado (2010), small to medium-sized species with 3 and 2 chaetae per bundle, common in

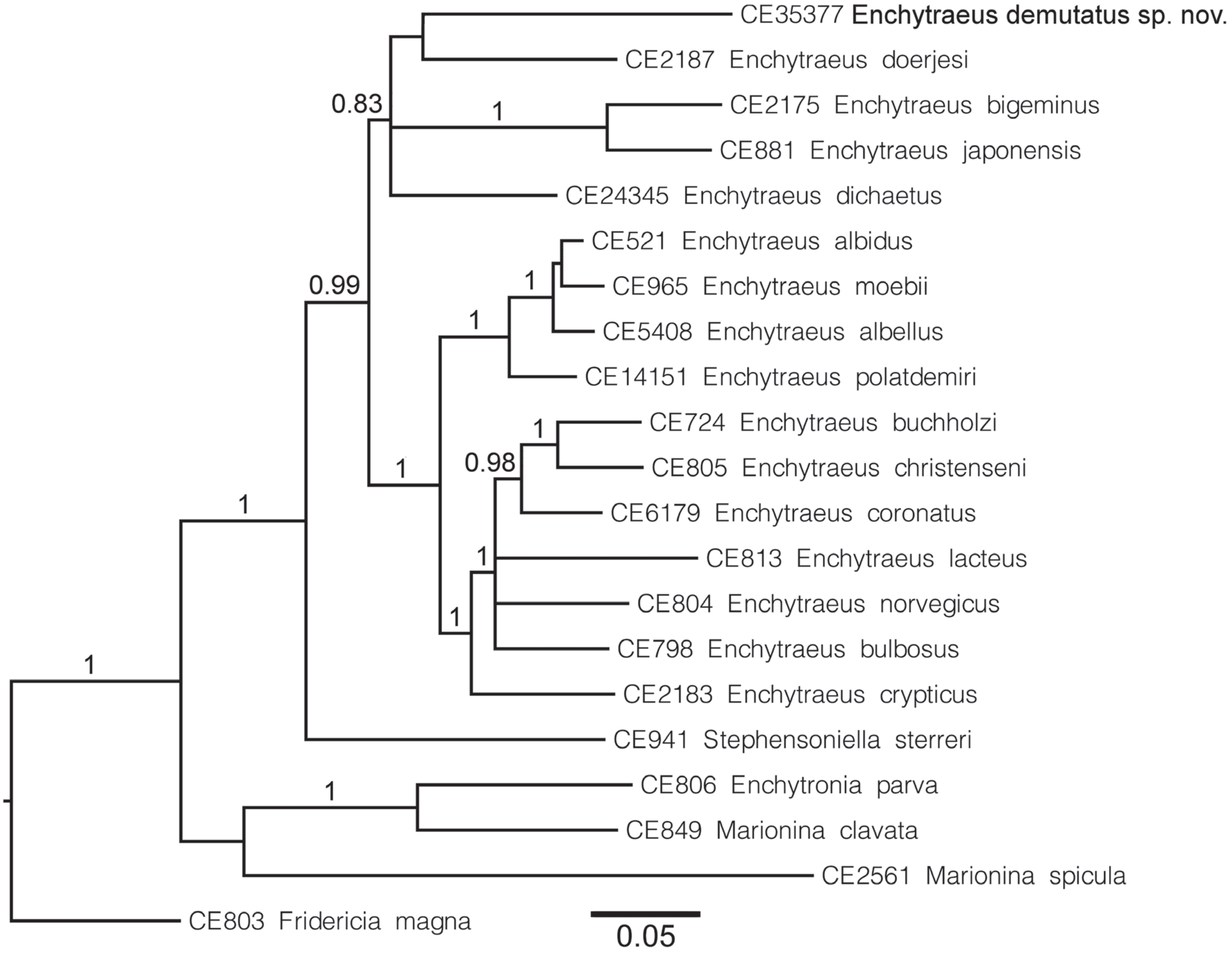

Figure 3. Phylogenetic tree based on a concatenation of six genetic markers, estimated using Bayesian inference. Posterior probabilities below 0.8 not shown. Scale bar indicates expected number of substitutions per site. For authorities of names see Table 1.

soils of well-studied Continental Europe. Even with fewer species included, it seems that the branch with *E. demutatus* is morphologically the more heterogeneous one in terms of morphology and reproductive modes.

Morphology, species comparison. Considering further peculiarities, the spermatheca with its elongate ectal duct, smooth outer surface, minute ectal glands and small ampulla is similar to the one in *E. doerjesi*, and possibly also to *E. indicus* Stephenson, 1912, even though here the ampulla is more than twice as wide as the duct (30 µm vs. 11–12 µm, Stephenson 1912: 239f.; there is no illustration). Interestingly, the latter species shares with *E. demutatus* the anterior placement of the first nephridium at 4/5. In other species of the genus, the first preclitellar nephridium is usually found at 6/7, or 7/8, 6/7 being by far the most common condition.

Most species of *Enchytraeus* have more than two chaetae at least in ventral preclitellar bundles. A pattern of 3 and 2 chaetae is most common in the smaller species, while most species of the *E. albidus* group have more than three in at least some bundles. One species, *E. przevalskyi* Hrabě, 1935 (from brackish Lake Issyk-Kul, Kirgistan), has only 1 chaeta per bundle, and one other species, *E. thomasi* Rodriguez & Giani, 1986 (from a brackish estuary in the Basque country, Spain), has up to 8 chaetae. Seven species are currently accomodated in *Enchytraeus* that share the strictly bisetose chaetal pattern with *E. demutatus*, *E. harurami* Stephenson, 1914, *E. parasiticus* Baylis, 1915, *E. bigeminus*, *E. varithecatus* Bouguenec & Giani, 1987, *E. japonensis*, *E. athecatus* Wang, Xie & Liang, 1999, and *E. dichaetus* (listed in chronological order). Apart from the oesophageal appendages and male efferent apparati, their differences to *E. demutatus* are as follows:

E. harurami Stephenson, 1914, described from a duckpond in Lahore, Northern India, and considered to be ‘thoroughly aquatic’ (Stephenson 1914: 335), has spermathecae that are not or only thinly attached to the oesophagus.

E. parasiticus Baylis, 1915, has 70–80 segments and masses of developing sperm in X and XI. Presence of testis sacs (as opposed to seminal vesicles, see Schmelz & Collado 2010: 29) is doubtful, and oesophageal appendages were not distinguished (‘No salivary glands have been seen’), therefore the position of this species in *Enchytraeus* is doubtful. The species was described from ‘the gill-chambers of the crab *Gecarcinus* [now *Johngarthia*] *lagostoma* in the island of south Trinidad’ (today referred to as Trindade Island, South Atlantic Ocean) (Baylis 1915).

E. bigeminus and *E. japonensis* are the two valid names within a complex of bisetose species that reproduce mainly by fragmentation and subsequent regeneration (Collado et al. 2012). Further differences (based on Bouguenec & Giani 1987, Schmelz et al. 2000): first nephridium at 8/9, often additional pharyngeal glands in VII; sexual specimens, when present, with forward-shift of the gonadal region by several segments, large testis sacs, and spermathecae with glandular ectal duct.

E. varithecatus Bouguenec & Giani, 1987, described from a compost heap in southern France, has large testis sacs and a ventrally interrupted clitellum.

E. athecatus Wang, Xie & Liang, 1999 from southern China lacks spermathecae and clitellar gland cells between the male pores.

E. dichaetus Schmelz & Collado, 2010, a replacement name for *E. minutus bisetosus* Rota & Healy, 1994, shares the girdle-shaped clitellum but often lacks spermathecae; when present, the ectal half of the ectal duct is covered with small glands.

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