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A new earthworm species and new records from Albania with remarks on the molecular phylogenetic relationships of the *Eisenia* species of the Balkan Peninsula (Oligochaeta: Lumbricidae)

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Abstract. Earthworm material collected from Albania has been elaborated. Altogether 11 species were found, of which *Lumbricus rubellus* Hoffmeister, 1843 represents a new record for the country and *Eisenia muranyii* sp. n. is new to science, raising the number of earthworm species present in Albania to 32. According to the results of the phylogenetic analysis of the different *Eisenia* species distributed in the Balkans, *E. muranyii* seems to be closest to *Eisenia lucens*.

Key words: Earthworms, Lumbricidae, Albania, new species, new records.

Introduction

The earthworm fauna of Albania is relatively well-known. In spite of the intensive soil zoology research in the Balkans in the last century, there were only sporadic data from this country (Pop 1943, Omodeo 1954, Bouché 1975). Recently, Dhora (2010) summarized the fauna of Albania and recorded the presence of altogether 19 earthworm species from the region.

Due to the collecting trips organised by the researchers of the Hungarian Natural History Museum to the Balkans in the last decade, the earthworm fauna of Albania has been enriched with several new data and two species new to science (Szederjesi & Csuzdi 2012), raising the number of earthworm species present in the country to 30.

Elaborating new material collected in 2012 and also some scattered samples from the previous years resulted in recording *Lumbricus rubellus* Hoffmeister, 1843 for the first time in Albania and discovery of a peculiar *Eisenia* species new to science.

To clear the phylogenetic position of this milk-white species within the usually purple-red genus *Eisenia*, phylogenetic analysis using partial COI gene sequences (barcode region; Hebert et al. 2003) were carried out. Because the so far published phylogenetic analyses of the lumbricid earthworm species (Briones et al. 2009, Pérez-Losada et al. 2012) did not contain data on the genus *Eisenia* apart from the debated *Eisenia fetida/andrei* species complex (Pérez-Losada et al. 2005, Blakemore 2013) to our knowledge, this is the first attempt to infer the phylogenetic relationships of this group of earthworms.

Materials and methods

Earthworms were collected by the diluted formaldehyde method (Raw 1959), complemented with digging and hand-sorting. The specimens were killed and fixed in 96% ethanol, then transferred into 75% ethanol and deposited in the earthworm collection of the Hungarian Natural History Museum (HNHM). For molecular studies, some specimens were placed into 96% ethanol. Sampling site characterizations and species found per site are given in Table 1.

Genial setae were removed by dorsal dissecting of the specimens and investigated using a Hitachi SN-2600N scanning electron microscope. For SEM studies they were glued on aluminum stubs using double-sided carbon tape and sputter-coated with gold.

Molecular studies on the different *Eisenia* species were carried out at the laboratories of the Department of Microbiology, Eötvös Loránd University and the Hungarian Natural History Museum. Parts of the muscular body wall behind the clitellum were cut and cleaned for analysis. DNA extraction was performed with Dneasy® Tissue Kit (Quiagen) according to the standard protocol. The primers 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′) were used for amplification of the COI gene with PCR. The DNA fragments were detected with agarose gel electrophoresis in 1% agarose. DNA purification was carried out with PCR-M™ Clean Up System (Viogene). The sequencing reactions were performed with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), using the primer HCO2198.

The chromatograms were corrigated with Chromas v1.45 (Technelysium Pty Ltd, Australia). Sequences were compared with the GenBank nucleotide database using blastn 2.2.14 algorithm (Altschul et al. 1997).

Table 1. Collecting sites and species found in Albania.

Region name	Alt. (a.s.l.)	Site description	Species found
Dibër	1330 m	karst forest, limestone rocks and dry grassland	*Ai. eiseni*
Dibër	1440 m	spring, stream and streamside vegetation	*D. byblica byblica*
Dibër	1770 m	spring and torrent	*D. byblica byblica*
Dibër	2165 m	large bog and its inflow torrents	*D. byblica byblica*
Dibër	2160 m	open streams	*D. byblica byblica*
Kukës	1430 m	open stream and alpine grassland	*Ap. caliginosa*
Kukës	1940 m	beech and pine forest	*D. alpina alpina, Dd. rubidus rubidus, L. rubellus*
Kukës	815 m	spring outlet in a secondary alder forest	*D. byblica byblica*
Kukës	1450 m	stream and its gorge	*Oc. transpadanus*
Kukës	1800 m	open brook and alpine grassland	*O. lacteum*
Malësi e Madhe	900 m	karst cave sidespring of the Cem River and its littoral vegetation	*Ap. smaragdina*
Skhodër	895 m	stream	*Dd. rubidus rubidus, L. rubellus*
Tiranë	1315 m	forest brook	*E. muranyii*
Tiranë	1495 m	limestone rocks and grassland	*D. attemsi*

Table 2. List of species analysed and GenBank Accession numbers.

Species	Accession number (GenBank)	Collecting locality
Eisenia fetida (Savigny, 1826)	FJ214228	-
Eisenia fetida (Savigny, 1826)	JX531566	-
Eisenia andrei Bouché, 1972	JN870085	-
Eisenia andrei Bouché, 1972	JN870086	-
**Eisenia spelaea* (Rosa, 1901)	KJ866411	Hungary, Sopron
**Eisenia spelaea* (Rosa, 1901)	KJ866412	Croatia, Papuk
**Eisenia lucens* (Waga, 1957)	KJ866407	Slovakia, Nízke Tatry
**Eisenia lucens* (Waga, 1957)	KJ866406	Romania, Oltenia
**Eisenia lucens* (Waga, 1957)	KJ866405	Hungary, Regéc
**Eisenia storkani* Černosvitov, 1934	KJ866413	Bulgaria, Stara Planina
**Eisenia ebneri* (Michaelsen, 1914)	KJ866404	Greece, Taigetos Mts.
**Eisenia oreophila* Szederjesi & Csuzdi 2012	KJ866409	Greece, Lakmos Mts.
**Eisenia oreophila* Szederjesi & Csuzdi 2012	KJ866410	Greece, Timfristos Mts.
**Eisenia muranyii* sp. n.	KJ866408	Albania, Gropë Mts.

* denotes new GenBank entries.

DNA sequences were aligned with ClustalW (Thompson et al. 1994), using the default settings. Phylogenetic analysis was carried out with MEGA 5.0 B (Kumar et al. 2008). The Neighbour Joining algorithm was used with Kimura-2-parameters model. A bootstrap analysis with 1000 pseudoreplicates was performed. Additional *Eisenia fetida* and *E. andrei* COI sequences were downloaded from GenBank (Benson et al. 2007) (Table 2).

Results

Allolobophoridella eiseni (Levinsen, 1884)
Lumbricus eiseni Levinsen, 1884: 241.
Allolobophoridella eiseni: Mršić 1991: 254., Dhora 2010: 82., Szederjesi & Csuzdi 2012: 262.

Material examined. HNHM/16162 3 ex., Dibër district, Lurë area, karst forest, limestone rocks and dry grassland along the Cidhnë–Fushë Lurë road, 1330 m, N41°48.892′ E20°16.650′, leg. L. Dányi, Z. Erőss, Z. Fehér, A. Hunyadi, D. Murányi, 29.06.2007.

Aporrectodea caliginosa (Savigny, 1826)
Enterion caliginosum Savigny, 1826: 180.
Aporrectodea (Aporrectodea) caliginosa caliginosa: Mršić 1991: 321.
Aporrectodea caliginosa: Dhora 2010: 82.

Material examined. HNHM/16161 1 ex., Kukës district, Turaj, open stream and alpine grassland along the Novoselë–Kolesjan road, NE of the village, 1430 m, N41°57.222′ E20°34.160′, leg. L. Dányi, Z. Erőss, Z. Fehér, A. Hunyadi, D. Murányi, 24.06.2007.

Aporrectodea smaragdina (Rosa, 1892)
Allolobophora smaragdina Rosa, 1892: 1.
Aporrectodea (Aporrectodea) smaragdina: Mršić 1991: 308.

Aporrectodea smaragdina: Szederjesi & Csuzdi 2012: 263.

Material examined. HNHM/16472 1 ex., Malësi e Madhe district, Madhë Mts., Gropa e Sëlces, karst cave sidespring of the Cem River and its littoral vegetation, S of the village, 900 m, N42°31.937′ E19°39.052′, leg. T. Deli, Z. Erőss, Z. Fehér, D. Murányi, 04.10.2005.

Dendrobaena alpina alpina (Rosa, 1884)
Allolobophora alpina Rosa, 1884: 28.
Dendrobaena alpina alpina: Mršić 1991: 627., Szederjesi & Csuzdi 2012: 264.

Material examined. HNHM/16157 1 ex., Kukës district, Gjalica e Lumës Mts., beech and pine forest W of the ridge, 1940 m, N41°59.974′ E20°27.464′, leg. L. Dányi, Z. Erőss, Z. Fehér, A. Hunyadi, D. Murányi, 25.06.2007.

Dendrobaena attemsi (Michaelsen, 1902)
Helodrilus (Dendrobaena) attemsi Michaelsen, 1902: 47.
Dendrobaena attemsi: Mršić 1991: 604., Szederjesi & Csuzdi 2012: 264.
Dendrobaena apora: Dhora 2010: 82.

Material examined. HNHM/16163 1 ex., Tiranë district, Gropë Mts, limestone rocks and grassland at Shtyllë Pass, along the Klos–Elbasan road, 1495 m, N41°22.270′ E20°05.126′, leg. L. Dányi, Z. Erőss, Z. Fehér, A. Hunyadi, D. Murányi, 30.06.2007.

Dendobaena byblica byblica (Rosa, 1893)
Allolobophora byblica Rosa, 1893: 4–5.
Dendrobaena byblica f. *typica*: Pop 1943: 20.
Dendrobaena byblica (part.): Mršić 1991: 566., Dhora 2010: 82.
Dendrobaena byblica byblica: Szederjesi & Csuzdi 2012: 265., Szederjesi *et al.* 2013: 394.

Material examined. HNHM/16156 16 ex., Dibër district, Korab Mts, Radomirë, spring, stream and streamside vegetation E of the village, 1440 m, N41°49.032′ E20°30.016′, leg. L. Dányi, Z. Erőss, Z. Fehér, A. Hunyadi, D. Murányi, 26.06.2007. HNHM/16158 1 ex., Kukës district, Lojme, spring outlet in a secondary alder forest, N of the village, 815 m, N41°59.898′ E20°31.393′, leg. L. Dányi, Z. Erőss, Z. Fehér, A. Hunyadi, D. Murányi, 24.06.2007. HNHM/16159 5 ex., Dibër district, Korab Mts., spring and torrent beneath Fushë Korabit, 1770 m, N41°49.207′ E20°30.727′, leg. L. Dányi, Z. Erőss, Z. Fehér, A. Hunyadi, D. Murányi, 28.06.2007. HNHM/16160 6 ex., Dibër district, Korab Mts, large bog and its inflow torrents beneath Mt. Korab, 2165 m, N41°48.229′ E20°33.642′, leg. L. Dányi, Z. Erőss, Z. Fehér, A. Hunyadi, D. Murányi, 27.06.2007. HNHM/16709 1 ex., Dibër district, Korab Mts., open streams beneath Mt. Korab, 2160 m, N41°47.971′ E20°33.475′, leg. T. Kovács, G. Puskás, 01.06.2013.

Dendrodrilus rubidus rubidus (Savigny, 1826)
Enterion rubidum Savigny, 1826: 182.
Dendrodrilus rubidus rubidus: Mršić 1991: 263., Szederjesi & Csuzdi 2012: 269.

Material examined. HNHM/16153 1 ex., Kukës district, Gjalica e Lumës Mts, beech and pine forest W of the ridge, 1940 m, N41°59.974′ E20°27.464′, leg. L. Dányi, Z. Erőss, Z. Fehér, A. Hunyadi, D. Murányi, 25.06.2007. HNHM/16474 1 ex., Skhodër district, Prokletije Mts., Okol, stream along the path towards Valbonë Pass, 895 m, N42°24.255′ E19°46.483′, leg. K. Balogh, Z. Barina, D. Murányi, D. Pifkó, 01.06.2005.

Eisenia muranyii **sp. nov.** (Figure 1, 2, 3)
Holotype: HNHM/16116, Tiranë district, Gropë Mts., Shëngjergj, forest brook along the road to Elbasan, E of the village, under decaying log, 1315 m, N41°19.800′ E20°08.739′, leg. P. Juhász, T. Kovács, D. Murányi, G. Puskás, 11.10.2012.

Figure 1. Habitus picture of living *E. muranyii* sp. n.

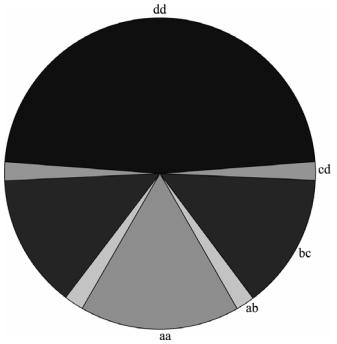

Figure 2. Setal arrangement of *E. muranyii* sp. n.

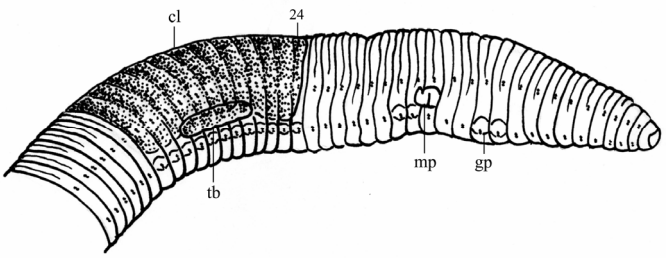

Figure 3. Ventrolateral view of the clitellar region of *E. muranyii* sp. n. cl = clitellum, gp = genital papilla, mp = male pore, tb = tubercle

Paratypes: HNHM/16117 10 ex., HNHM/16220 1 ex., locality and date same as that of the Holotype.

Etymology: The new species is named in honour of our colleague Dr. Dávid Murányi who collected valuable earthworm materials for us including the new species.

Diagnosis: Length 48–91 mm, diameter 4–5 mm, setae closely paired. Pigmentation lacking. First dorsal pore in 5/6. Clitellum on ½ 24–35, tubercles on 28–1/n 33. Male pore on 15, large. Nephridial pores irregularly alternate between setal line *b* and above *d*. Four pairs of vesicles in 9–12; spermathecae 9/10, 10/11 in *d*. Calciferous glands in 11–12 with well-developed diverticulum in 12. Hearts in segments 7–11. Nephridial bladders simple, sausage-shaped.

External characters: *Holotype:* 91 mm long and 5 mm wide. Number of segments 110. *Paratypes*: 48–81 mm long and 4–5 mm wide. Number of segments 90–127. Pigmentation lacking, colour pale conserved but milk-white alive (Fig. 1). Prostomium epilobous ½ open. First dorsal pore at intersegmental furrow 5/6. Setae closely paired. Setal arrangement behind clitellum: *aa*:*ab*:*bc*:*cd*:*dd* = 8.6:1:7.3:1:24.6 (Fig. 2). Male pores on segment 15, surrounded by a glandular crescent, protruding into segment 16. Nephridial pores irregularly alternate between setal line *b* and above *d*. Clitellum on segments ½ 24–35. Tubercula pubertatis on segments 28–1/n 33. Genital papillae on segments 11, 12, 16, 17, 24–35 *ab* (Fig. 3).

Internal characters: Septa 6/7–12/13 slightly thickened. Testes and funnels paired in segments 10–11, covered with perioesophageal testis sacs. Seminal vesicles in 9–12, the vesicles in 10 are somewhat smaller than the others. Spermathecae in 9/10, 10/11 with external openings in setal line *d*. Calciferous glands in 11–12 with well-developed diverticula in segment 12. Paired hearts appear in segments 7–11, with a pair of small extraoesophageal vessels in 12. Nephridial bladders simple, sausage-shaped. Crop in segments 15–16, and gizzard in segments 17–18. Typhosolis T-shaped. The cross-section of the longitudinal muscle layer is of fasciculated type.

Remarks. The new species resembles *Eisenia lucens* (Waga, 1957) and *E. spelaea* (Rosa, 1901) in the position of the clitellum (½ 24–35 vs. 25, 26, 27–33, 34), tubercula pubertatis (28–1/n 33 vs. ½ 28, 28, 29–31, 32) and the number of vesicles. However, the new species differs from both in the position of the clitellum, pigmentation and presence of calciferous diverticulum in 12. *Eisenia muranyii* sp. nov. is similar also to a set of Central Asian species but differs from them by the fasciculated musculature and other characters (Table 3).

Lumbricus rubellus Hoffmeister, 1843

Lumbricus rubellus Hoffmeister, 1843: 187., Mršić 1991: 474.

Material examined. HNHM/16152 1 ex., Kukës district, Gjalica e Lumës Mts, beech and pine forest W of the ridge, 1940 m, N41°59.974'

Table 3. Distinguishing characters of *E. muranyii* sp. n. and the similar *Eisenia* species from the Balkan and Asia.

	Clitellum	Tubercles	Spermathecae	Calciferous diverticulum	Musculature type
E. lucens (Waga, 1857)	25, 26, 27–33, 34	½ 28, 29–31, 32	9/10, 10/11 M–d	–	fasciculate
E. spelaea (Rosa, 1901)	25, 26, 27–33, 34	28, 29–31, 32	9/10, 10/11 d–M	–	fasciculate
E. gordejeffi Michaelsen, 1899	23, 24–34. 35	27, 28–31	9/10, 10/11 M	11, 12	pennate
E. lagodechiensis (Michaelsen, 1910)	24, 25–35	28, 29–32	9/10, 10/11 M	12	pennate
E. djungarica (Perel, 1969)	26, 27–34, 35	29–32	9/10, 10/11 d–M	11, 12	pennate
E. muranyii sp. nov.	½ 24–35	28–1/n 33	9/10, 10/11 d	12	fasciculate

E20°27.464′, leg. L. Dányi, Z. Erőss, Z. Fehér, A. Hunyadi, D. Murányi, 25.06.2007. HNHM/16473 1 ex., Skhodër district, Prokletije Mts., Okol, stream along the path towards Valbonë Pass, 895 m, N42°24.255′ E19°46.483′, leg. K. Balogh, Z. Barina, D. Murányi, D. Pifkó, 01.06.2005.

Remark. Though this peregrine species hasn't been reported so far from Albania, this record was expected as *L. rubellus* is spread all over the Balkans.

Octodrilus transpadanus (Rosa, 1884)
Allolobophora transpadana Rosa, 1884: 45.
Octodrilus transpadanus: Mršić 1991: 371., Dhora 2010: 82., Szederjesi & Csuzdi 2012: 270.

Material examined. HNHM/16155 1 ex., Kukës district, Turaj, stream and its gorge at the village, 1450 m, N41°56.298′ E20°33.480′, leg. L. Dányi, Z. Erőss, Z. Fehér, A. Hunyadi, D. Murányi, 24.06.2007.

Octolasion lacteum (Örley, 1881)
Lumbricus terrestris var. *lacteus* Örley, 1881: 584.
Octolasion tyrtaeum: Mršić 1991: 347.
Octolasion lacteum: Dhora 2010: 82.

Material examined. HNHM/16154 1 ex., Kukës district, Turaj, open brook and alpine grassland along the Novoselë–Kolesjan road, E of the village, 1800 m, N41°56.594′ E20°29.879′, leg. L. Dányi, Z. Erőss, Z. Fehér, A. Hunyadi, D. Murányi, 24.06.2007.

Discussion

Out of the 11 recorded species, *Lumbricus rubellus* Hoffmeister, 1843 represents a new record to the fauna of Albania and *Eisenia muranyii* sp. n. is new to science. With these data, the number of earthworm species recorded for Albania is raised to 32.

According to the results of the phylogenetic analysis of the different *Eisenia* species, three main clades can be distinguished (Fig. 4). The first clade consists of *E. lucens* (Waga, 1857), *E. muranyii* sp. n. and *E. spelaea* (Rosa, 1901). Therefore, despite the lack of pigmentation, the new species seems to be closest to *Eisenia lucens* but connected to it with a very long branch and low bootstrap support (41%) which indicates that this placement is not stable. However, there are similarities also in several morphological characteristics such as the fasciculate longitudinal musculature and in the cross-section trihedral genital setae (Fig. 5–6). *E. lucens* is a widely distributed Carpathian-Alpine species (Szederjesi et al. 2014) found usually under bark of decaying logs. The new species was also found under decaying wood.

The second main clade is composed of the *Eisenia fetida* (Savigny, 1826) and *E. andrei* Bouché, 1972. The species of the *Eisenia grandis* group (Zicsi & Csuzdi 1986, Szederjesi 2013) formed the well-separated third clade. This clade differs also in the morphology of the genital setae which are tetrahedral in cross section (Fig. 7). On the basis of this

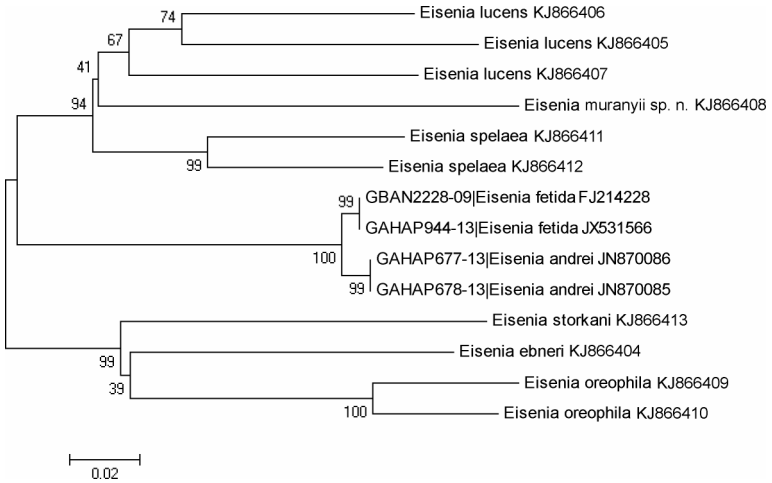

Figure 4. Neighbour Joining tree of the Balkanic *Eisenia* species.

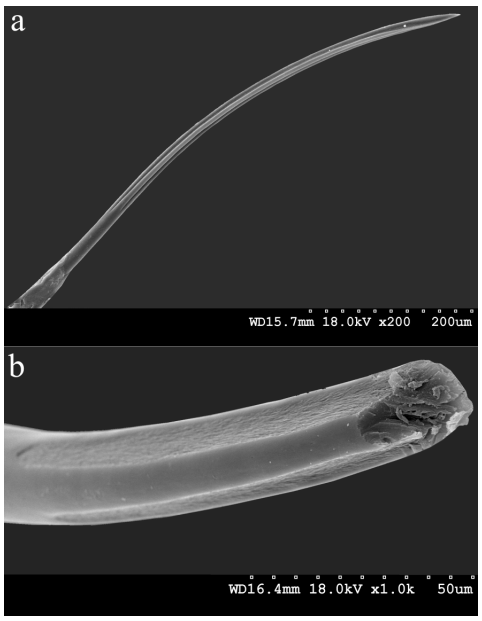

Figure 5. *Eisenia muranyii* sp. n., genital seta.
a = full length seta, b = cross section.

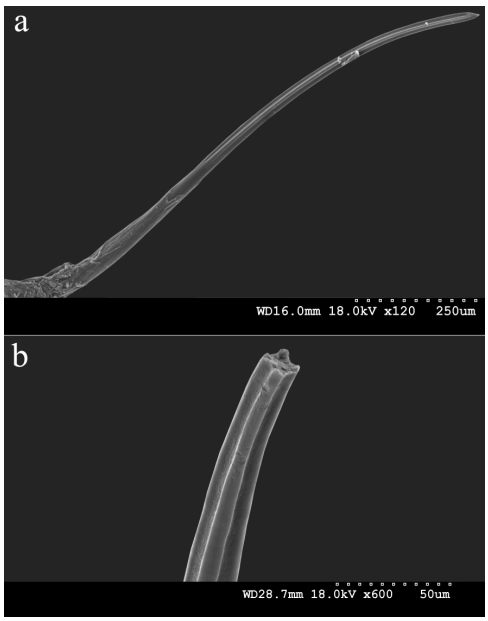

Figure 7. *Eisenia oreophila,* genital seta.
a = full length seta, b = cross section.

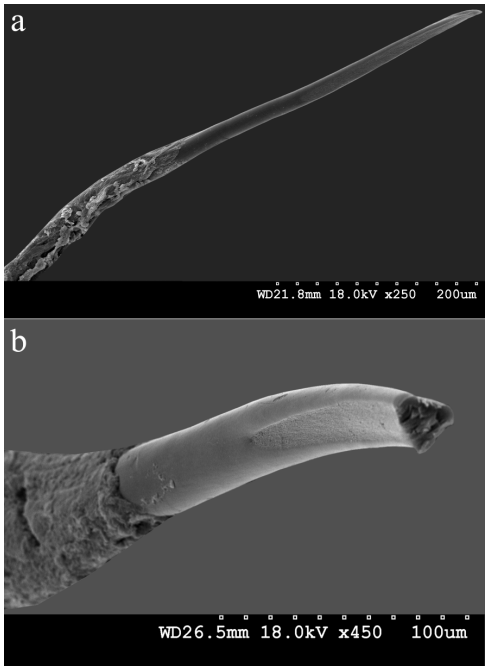

Figure 6. *Eisenia lucens,* genital seta.
a = full length seta, b = cross section.

character Kvavadze (2000) separated *E. grandis* and the related species into a new genus *Dendrodriloides* Kvavadze, 2000, however, further studies are needed to determine the validity of this genus.

The interspecific genetic distances (K2P) between the investigated Balkanic *Eisenia* species are all above 15% proposed by Chang & James (2011) for delimiting earthworm species except the highly disputed *E. fetida* – *E. andrei* species pairs (see Blakemore 2013) which show only 1.3% K2P genetic distance (Table 4).

Table 4. K2P genetic distances of the Balkanic *Eisenia* species.

	1	2	3	4	5	6	7
E. fetida							
E. andrei	0.013						
E. spelaea	0.199	0.198					
E. lucens	0.223	0.220	0.184				
E. storkani	0.230	0.242	0.249	0.263			
E. ebneri	0.225	0.234	0.248	0.249	0.199		
E. oreophila	0.243	0.251	0.262	0.258	0.214	0.200	
E. muranyii sp. n.	0.246	0.248	0.206	0.216	0.282	0.257	0.288

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