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Green light to an integrative view of *Microscolex phosphoreus* (Dugès, 1837) (Annelida: Clitellata: Acanthodrilidae)

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Abstract

The small synanthropic and peregrine earthworm *Microscolex phosphoreus* (Dugès, 1837) is reported for the first time from Siberia. Morphological and DNA barcode (COI) analyses of this and widely separate samples worldwide demonstrate that, as currently identified, *M. phosphoreus* is a heterogeneous taxon, with divergent lineages occurring often in the same locality and hardly providing geographically structured genetic signals. The combined morphological and genetic evidence suggests that at least four of the found clades should be reclassified as separate species, both morphologically and genetically distinct from each other. However, as the specimen number was limited and only the COI gene was studied for the genetic work, we hesitate in formally describing new species. There would also be the problem of assigning the available names to specific lineages. Our findings encourage careful external and anatomical examination and using reliable characters such as the interchaetal distances and spermathecal morphology for correct identification and for deeper evaluation of cryptic diversity in this interesting bioluminescent worm.

Keywords: peregrine earthworm, cryptic diversity, morphology, DNA-barcoding, bioluminescence

Introduction

Microscolex phosphoreus (Dugès, 1837) is a small synanthropic earthworm, remarkable for its bioluminescence, with a circummundane distribution in subtropical and warm temperate regions (see Rota 2013). It is classified in the Acanthodrilidae and, like other species in this megascolecoid family, shows a reduction of the male apparatus (the posterior prostates have disappeared and the male pores tend to be found close to the prostate pores in XVII), combined with a weak gizzard and vesiculated nephridia. All available descriptions give a rather consistent picture of its external and internal organization, except for two aspects: one somatic, i.e. the interchaetal spaces, the other reproductive, i.e. the structure and location of the spermathecae (see below).

The worm is mostly found in near-coastal areas, but in Europe it has spread as far inland as deep coal mines in southern Poland (Rota & de Jong 2015). In Asia, it is known from Israel, Turkey, Iran, Pakistan and India and has been widely recorded in Japan, up to 38°16'N (Oba *et al.* 2016). This part of Japan has snowy winters but low temperatures never below -5°C. In all of Russia there was so far only one record, from nearby rural buildings in Khosta Microdistrict, on the eastern coast of the Black Sea (Perel 1979; Vsevolodova-Perel 1997), but two of us (V.N. Petushkov & N.S. Rodionova) recently discovered a vigorous population of bioluminescent *Microscolex* in a small village by the Lake Bajkal, Siberia (51°54'N).

Stimulated by this finding and by the unusual traits of the Siberian worms (dorsal interchaetal distance *dd* short; spermathecal ampullae large and with minute diverticula), we set to investigate whether *M. phosphoreus* is a single, polymorphic species or a complex of (possibly cryptic) species by using morphological and molecular

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markers. We surveyed the chaetal arrangement and spermathecal features in a reference collection of *M. phosphoreus* originating from Australia, North Africa, Spain and Italy. At the same time, we analysed new (from Siberia, Australia, Spain) and available (from Japan, South Africa, France, Israel and USA, in GenBank and BOLD public libraries) cytochrome c oxidase subunit I (COI) mitochondrial gene sequences of *M. phosphoreus* sensu lato, aiming at verifying the congruence between the morphological and molecular patterns. Our study presents the first combined morphological-molecular study covering a large part of the geographical range of *M. phosphoreus*.

Nomenclatural and biogeographic backgrounds. *Microscolex phosphoreus* was the first species of luminous clitellate worms ever described (Rota 2009). It was Antoine Dugès (1837), at the dawn of earthworm taxonomy, who first attributed a specific rank to this animal. His *Lumbricus phosphoreus* was discovered in the tanbed of a hothouse in the Jardin des Plantes de Montpellier, southern France. The worm (1.0–3.5 by 0.1–0.2 cm, semitransparent, red-blooded, with eight chaetae per segment and clitellum in XIII–XVI) emitted luminous fluid from the surface of the body, "a fluid no doubt similar to that released through the dorsal pores of many other worms". The species was classified in *Lumbricus*, although it differed from all known "lombrics" precisely because of the lack of dorsal pores and for the more frontal position of the clitellum.

Fifty years later, within months from one another, three non-lumbricid and supposedly non-autochthonous earthworm taxa were independently described, respectively, in northern Italy, in northern France and in the southeast of Australia: Rosa (1887) established *Microscolex modestus*, new genus and new species, for a worm found in terrariums and flowerpots in Turin and Genoa. Giard (1887) gave an extensive account of a luminous worm discovered in greenhouse potting soil in Wimereux, a worm that he recognized as possibly identical with Dugès' *phosphoreus* and for which he established the genus *Photodrilus*. Fletcher (1887) described a species of uncertain affinities, *Eudrilus? dubius*, from Sydney and Adelaide, specifying that it was only found in gardens. Rosa (1888) promptly discussed the similarities between these taxa and, although perplexed by some incongruences (*Photodrilus* had been erroneously described by Giard as possessing nephridia from XIV and male pores in XVIII, making it appear intermediate between the genera *Pontodrilus* and *Microscolex*), he advanced the hypothesis that they might be all synonyms. Shortly afterwards Rosa (1890) resolved that they should be reclassified as two congeneric species of *Microscolex*, one of which being luminescent, and that their true homeland was probably Argentina, as they had been observed there abundantly among the grass roots in all meadows (see more details in Rota 2009). Michaelsen (1899) reexamined Giard's material and, based on priority of publication, formally established the correct name of *M. phosphoreus*: *Lumbricus phosphoreus* Dugès, 1837 = *Microscolex modestus* Rosa, 1887 = *Photodrilus phosphoreus* (Dugès) (Giard 1887) = *Pontodrilus phosphoreus* (Dugès) (Beddard 1895) = *Microscolex phosphoreus* (Dugès) (Michaelsen 1899).

Later Michaelsen (1907: 148) further elaborated the synonymy of *M. phosphoreus*, lumping together seven more nominal species (*Microscolex algeriensis* Beddard, 1892, *M. novae-zelandiae* Beddard, 1893, *M. hempeli* Smith, 1896, *M. horsti* Eisen, 1900, *M. parvus* Eisen, 1900, *Deltania troyeri* Eisen, 1893, *D. benhami* Eisen, 1893). He regarded *M. phosphoreus*, as well as *M. dubius* (Fletcher), as circummundane and peregrine members of an otherwise subantarctic circumpolar genus (with 20 species confined to subantarctic latitudes). Michaelsen explained this widespread overseas distribution as related to the euryhaline capacity of the genus, as shown by frequency of records in littoral localities: "owing to its euryhaline nature the genus was driven from station to station by sea, as a result of the subantarctic sea current encircling the southern polar region, the so-called west wind drift, and thus became circumpolar". Certainly, in what concerns in particular *M. phosphoreus*, human agency (plant trade, ship ballast, etc.) during the last centuries, combined with an extraordinary invasive capacity and adaptability, was also very important, as suggested by the lack of published records of this common luminous worm in earlier times (Rota 2009). This implies that the geographic source of the studied material has a relative significance, since this peregrine morphospecies may have colonized on several occasions and from many places of origin one same region.

Morphological variability. In the literature, all descriptions of *M. phosphoreus* are consistent in reporting the upper and lower chaetae as being widely paired and the interval *ab* as being the smallest and *dd* the largest. However, the ratio between the midlateral (*bc*) and midventral (*aa*) intervals is reported differently from source to source. According to Rosa (1887), *bc>aa=cd*. According to Michaelsen (1900), *bc=aa>cd*; the same is reported by Díaz Cosín & Moreno (1979). According to Stephenson (1914), followed by Gates (1972), *bc>aa>cd*. According to Bouché (1972), *aa>bc>cd*. Yamaguchi (1935) observed that *ab<cd<bc*, *cd<aa<dd*; *bc<=aa* in preclitellar region, while *bc>aa* in postclitellar region.

Giard (1887) described the spermathecae as one pair in IX, opening in 8/9 on line *a*. Rosa (1887, 1888) stated the same for *M. modestus*, and so did Beddard (1892, as *M. algeriensis*; 1893, as *M. novaezelandiae*), Stephenson (1914), Yamaguchi (1935), Pickford (1937), Díaz Cosín & Moreno (1979) and Talavera & Pérez (2009). Lee (1959) for New Zealand—inexplicably (since he officially built his diagnosis from the descriptions by Beddard (1893), Michaelsen (1900) and Pickford (1937)—described the pores in a normal position but the spermathecae as located in VIII, a location stated also by Dyne & Jamieson (2004) for material from Australia and Tasmania. Also Csuzdi (1986) described specimens from Hungary and Balkans with spermathecae in VIII.

Giard (1887) mentioned that each spermatheca had a "petit sac accéssoire, comme *Pontodrilus*". Rosa (1888) described the latter as a lateral caecum, and later (1890) as a pipe-like diverticulum bulging a bit at the ends. Beddard (1892) in *M. algeriensis* noted that each spermatheca consisted of an oval pouch and a single narrow diverticulum opening into it in front, whereas in *M. novaezelandiae* each spermatheca had two diverticula, one directed anteriad and the other posteriad (Beddard 1893). Stephenson (1914), in *M. phosphoreus* from Northern India, found the ampulla to be pear-shaped and bearing two short diverticula arising separately or by a common stalk from the duct (also in same individual). Yamaguchi (1935) described, for Japanese individuals, each spermatheca as formed by a large main sac and a spherical diverticulum. Pickford (1937) noted variation among different South African localities: the ampulla could be rounded or pear-shaped, the duct short or as long as ampulla; the stalked, club-shaped diverticula could be one or two; in the latter case, they arised separately (she figured them as one medial and one lateral). Omodeo (1952) reported variable spermathecal morphology, even intraindividually, in worms from Turkey, with each spermatheca having one or two (arising by a common stalk) diverticula, and the diverticula sometimes being bilobed; he found the same variation in specimens from Naples, Italy. Lee (1959) reported a single, short, club-shaped medial diverticulum. Bouché (1972) figured the spermatheca as club-shaped and with "two bilateral diverticula". Gates (1972) reported the spermathecae as small and suboesophageal, and each ampulla as long or longer than duct, with two equal or subequal diverticula with ellipsoidal to ovoidal seminal chamber and united ectally to open into anterior face of duct". Csuzdi (1986) described club-shaped spermathecae, each with two, rarely one diverticulum. Blakemore (1994) described (material from south-eastern Australia) the spermathecae as "in IX, small, with pearshaped ampulla and two (occasionally only one) iridescent diverticula opposed on short duct".

Material and methods

Specimens included in the morphological study. The Siberian specimens of *M. phosphoreus* originate from soil samples collected in August 2016 in vegetable patches inside and immediately outside greenhouses at Bolshie Koty (51°54'23.5"N, 105°4'32.1"E), a former gold-mining site now belonging to Pribaikalsky National Park, near Listvyanka, 70 from Irkutsk, on the western shore of Lake Bajkal. They were discovered while studying co-occurring bioluminescent enchytraeids in samples carried to the Laboratory of Photobiology, Institute of Biophysics SB RAS, Krasnoyarsk, Russia (Rota *et al.* submitted; see also Rodionova *et al*. 2017). The luminescent activity of worms was measured using a custom-made luminometer (Oberon K, Krasnoyarsk, Russia). The self-luminous photograph in Fig. 1C was taken by a Panasonic, Lumix DMC-LX7 camera (f/1.4, 5 sec, ISO 6400). Live and alcohol preserved specimens were sent to Italy for identification and further taxonomic evaluation. The worms were examined under a Zeiss Stemi SR stereomicroscope and morphometric measurements were taken with a graticule eyepiece. Genital chaetae were removed from the inner wall of dissected adult worms. Caudal fragments were sent to Sweden for genetic analysis. All studied material is deposited in Omodeo & Rota's collection.

For comparisons, the following material in Erséus' collection (all collected by hand-sorting and DNA-sequenced) was examined: four specimens from Western Australia (CE16796–CE16799; Appendix 1), and one from Valencia, Spain (CE5290; Appendix 1). Morphological observations were also carried out in reference material from Omodeo & Rota's collection: **Morocco**, Mc 124, Prov. Khenifra, Mrirt/Khenifra (P24), sand from stream among arid pastures, 33°4'59.77"N, 5°35'21.73"W, 7.3.1981, P. Omodeo & G.B. Martinucci leg. Mc125, Prov. Béni-Mellal, Zaouia ech Cheikh (P24), steppe plain with cultivated fields, 32°39'12.15"N, 5°54'12.24"W, 7.3.1981, P. Omodeo & G.B. Martinucci leg. **Spain**, Catalonia, 9 km W of Girona, between Anglès and Bescano (N141), bank of River Ter, 41°58'29"N, 2°41'36"E, 30.1.1989, G. Bonifazi, P. Omodeo & M. Sciarra leg. **Italy**, Sardinia, stn. Ca2, 2 km ESE of Guamaggiore, 39°33'8.17"N, 9°4'36.21"E, stony pasture, 180 m a.s.l., 25.4.1980, P. Omodeo & C. Dattena leg. Apulia, Porto Selvaggio (Nardò, Lecce Province), 40°8'48"N, 17°58'15"E, 29.3.1996, D. Ferreri leg. Apulia,

Averni, Baia Verde (Gallipoli, Lecce Province), 40°2'13"N, 18°0'56"E**,** 23.3.1996, D. Ferreri leg. Sicily, Aeolian Islands (Messina Province), Vulcano, Vulcano Piano, 38°23'9.51"N, 14°58'35.36"E, 13.1.1986, M.G. Filippucci leg. Whenever possible, observations were validated by examining at least 2–3 specimens per site.

Molecular data. Details of all specimens included in the molecular study can be found in Appendix 1. None of the old reference material from Omodeo & Rota's collection listed above was included, due to fixation in denatured alcohol. The GenBank COI (barcode) sequences identified as *M. phosphoreus* originate from one main geographic source: Japan (Oba *et al.* 2011a,b, 2015, 2016, Oba 2012), representing specimens from throughout the country. From the same database we mined seven unidentified *M. phosphoreus* COI sequences, all from South Africa and bearing the label "Oligochaeta sp." (Voua Otomo *et al.* 2013). Another five mislabeled sequences were retrieved from Barcode of Life Data System (BOLD; accessed 28 July 2017): one, from the north of France, is misclassified as "Enchytraeidae"; a second, from Texas, USA, is misidentified as "*Diplocardia bichaeta*" (in Damoff 2008); two more sequences from Texas and the last, from Israel, are labeled as unspecified "Haplotaxida". To the publicly available sequences, we added nine newly obtained COI sequences from *M. phosphoreus* specimens collected by us in Siberia (1 spm), Western Australia (7 spm), and Spain (1 spm) and, as an outgroup, the sequences of two new specimens of *M. dubius* from Australia (Appendix 1).

DNA was extracted from a small piece of body wall from the posterior part of each specimen, using Epicentre's QuickExtract DNA Extraction Solution 1.0. The mitochondrial gene COI was amplified using the primer pair LCO1490/HCO2198 (Folmer *et al.* 1994). The sequences were assembled into consensus sequences using Geneious v.7.1.8 (Biomatters Ltd., Auckland, New Zealand), and were aligned using MAFFT (Katoh *et al*. 2002) as implemented in Geneious. A total of 80 sequences were included in the analysis, and the resulting alignment was 617 bp long. A phylogenetic tree was estimated using PhyML 3.0 (Guindon *et al*. 2010), as implemented at the Montpellier Bioinformatics platform (http://www.atgc-montpellier.fr/). The Smart Model Selection (Lefort *et al*. 2017) with Bayesian Information criterion was used for automatic model selection; Subtree Pruning and Regrafting were used for tree improvement. Branch support was calculated with the SH-like (Shimodaira-Hasegawa test-like) approximative likelihood ratio test (aLRT) (Anisimova & Gascuel 2006). For each main group of *M. phosphoreus* with more than one sequence (see results), a haplotype network was calculated in PopArt v 1 (Leigh & Bryant 2015) using statistical parsimony (Templeton *et al.* 1992; Clement *et al.* 2002). Further, the minimum uncorrected pairwise genetic distances between the clades, and the maximum distances within groups were calculated in MEGA 7 (Kumar *et al.* 2016).

Results

Morphology of Siberian specimens. Body length *in vivo* 35–55 mm, width 0.8–1.8 mm at clitellum; size after fixation up to 47 by 1.7 mm. Segments (63) 73–79. Unpigmented (Fig. 1D,F), blood vessels showing *in vivo* through transparent body wall; clitellum opaque, yellow to orange (Fig. 1A,B,D,E), white when worms swim in water.

Prostomium epilobous, open, 1/3–2/3. Setae 4 pairs per segment; *ab*<*bc*<*cd*<*aa*<*dd* preclitellarly, *ab*<*cd*<*aa*<*bc*<*dd* postclitellarly. Average chaetal distances at XXVI (n = 5) *aa: ab: bc: cd: dd* = 1.41: 1.0: 1.55: 1.09: 1.69; the distance *dd* behind the clitellum is short, about 1/6 of the body circle (Fig. 1E). Chaetae *ab* closer to midventral line in clitellar segments (Fig. 1D) and those of XVII modified as elongate (l=450 µm, width at midlength 10 µm), almost capillary, sinuous genital chaetae with small knobbed ectal end (Fig. 2C), emerging as a narrow couple near line *a*. Dorsal pores absent. Clitellum annular, ½XIII, XIII–½XVII, XVII, incomplete ventrally on the first and last segments. Nephropores of II–IV in lines *d*; then in *c*, conspicuous on clitellum (Fig. 1D,F). Female pores on lines *a*, in anterior half of XIV (Fig. 1D). Male pores and prostatic pores paired on XVII, opening close to each other, in front of and laterally to the modified genital chaetae *b*, respectively. Genital papillae (visible in dried worms and in detached cuticle) paired in XVII by the male pores and (sometimes unpaired) in XVIII on line *b*. Spermathecal pores paired, inconspicuous, in 8/9, line *a*.

Septa thickened 7/8<8/9–12/13>13/14–14/15 (Fig. 2A). Rudimentary gizzard in V. Calciferous glands absent. Intestine commencing in XVI; typhlosole absent. Dorsal blood vessel single; hearts in X, XI and XII (Fig. 2A), slightly increasing in size posteriad. Nephridial vesicles small anteriorly; posteriorly large, shaped like a transverse ocarina, oriented with bluntly rounded end dorsally and the pointed end ventrally; the latter receives the tubular portion of the nephridium; laterally, the funnel-shaped mouthpiece narrows to nephropore. Holandric, testes and

funnels paired in X and XI. Seminal material free and included in small paired seminal vesicles in XI–XII. Ovaries made of egg strings, in XIII. Prostates one pair, S-shaped, occupying one or two segments, XVII, or XVII and XVIII (Fig. 2A). Spermathecae one pair, ampullae (full of sperm) occupying whole length of IX, each resembling a large cabbage surrounded by a blood capillary vessel; duct short, with single, knobstick-like (sometimes bilobed), medial or anterior diverticulum (Fig. 2A,B).

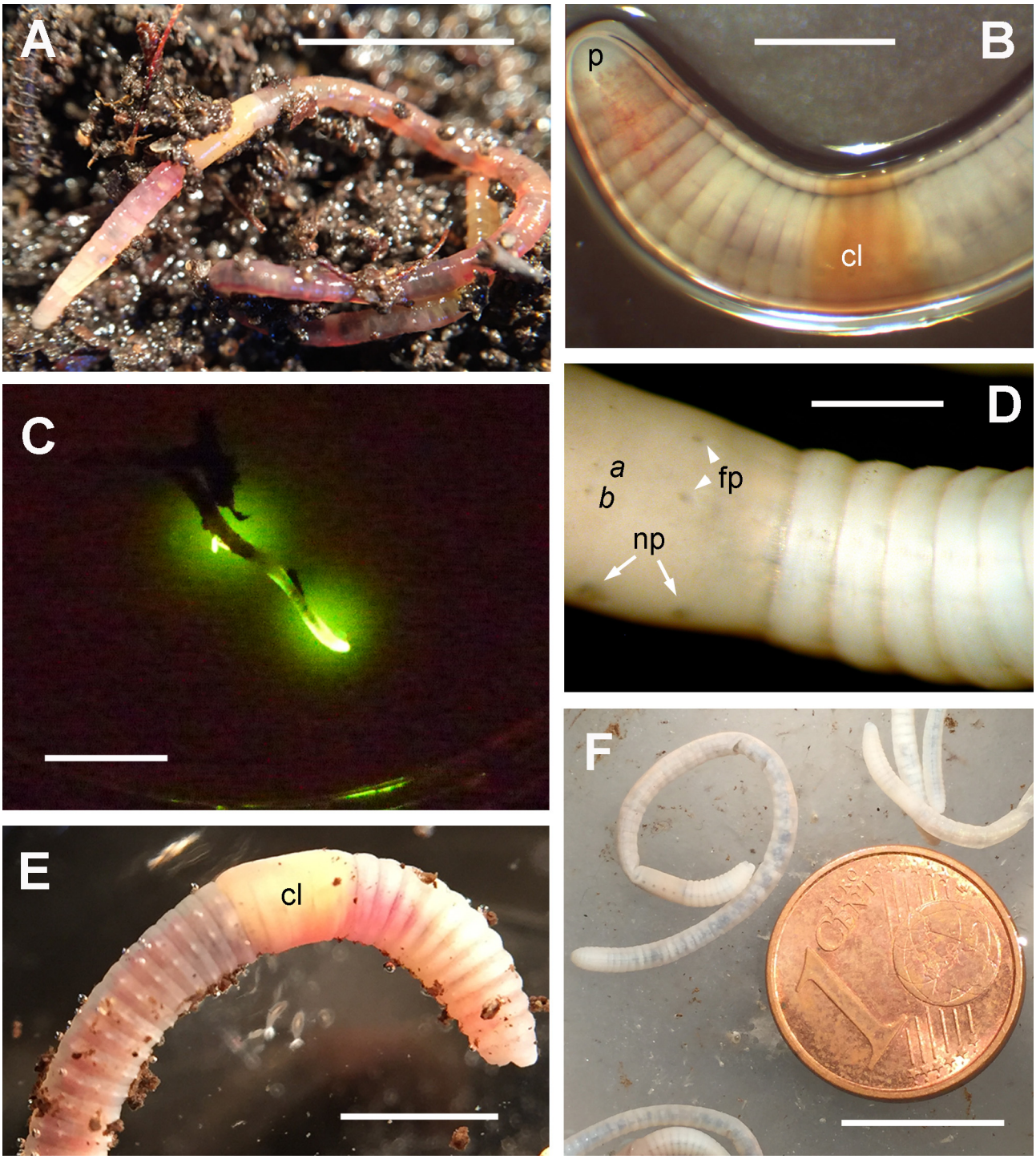

FIGURE 1. Morphology of *Microscolex phosphoreus* (Dugès, 1837) from Bolshie Koty, Siberia. **A.** Live adult specimen in soil. **B.** Anterior body region (anesthetized worm compressed between slides; lateral view), showing the rich parietal vascularization (cl= clitellum; p= prostomium). **C.** Self-luminous worm after stimulation with ethanol. **D.** Ventral view of segments VIII–XV, showing the female pores (fp) in XIV, the closely paired chaetae *ab* and the conspicuous nephropores (np) on line *c* of clitellar segments. **E.** Anterior body half at an early stage of ethanol-fixation, showing the high position of dorsal chaetae behind clitellum (cl). **F.** Facies of adult worms after fixation. Scale bars: A, C, F = 10 mm; B = 2 mm; D = 1 mm; E = 3 mm.

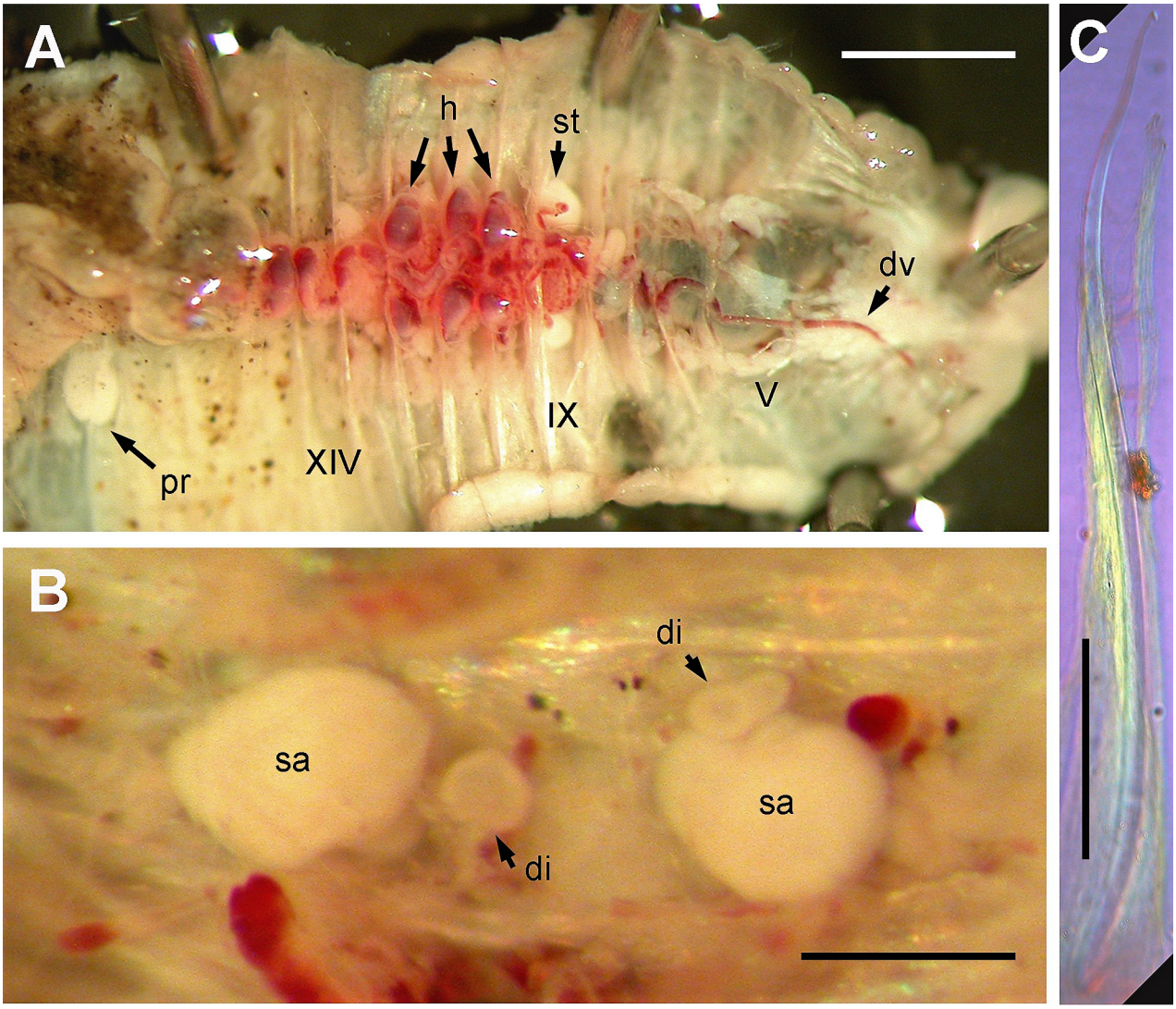

FIGURE 2. Anatomy of *Microscolex phosphoreus* (Dugès, 1837) from Bolshie Koty, Siberia. **A.** Internal view of a dorsally dissected adult specimen (dv= dorsal blood vessel; h= hearts; pr= prostate; st= spermatheca). **B.** Close-up of the spermathecae (posterior view) on the floor of segment IX, after removal of the alimentary canal. Note the large ampullae (sa) and the comparatively small single, knobstick-like diverticula (di), arising medially in the left spermatheca, and anteriorly (and with bilobed head) in the right spermatheca. **C.** Genital chaeta *b* of XVII. Scale bars: A = 1 mm; B = 300 µm; C = 100 µm.

Light production by Siberian specimens. The Siberian *Microscolex* worms emit bright green bioluminescence (Fig. 1C), with a maximum at 530 nm (measured *in vivo*). When undisturbed, the worms practically do not shine, nor weak tactile stimulations are sufficient to make them glow: coelomic fluid (the site of luminescence) is discharged only following strong mechanical, electrical or chemical irritation. The maximum intensity of luminescence depends on the amount of coelomic fluid discharged; therefore, the glowing of juveniles is noticeably weaker than that of adult specimens. Luminescence *in vivo* lasts for a few minutes and the dynamics of decay is much disturbed by the worm's movements which interfere with the release of the system components. However, even when the worm is placed in 80% ethanol, which practically paralyses it after 3 minutes, the luminescence goes through many fluctuations. When isolated, the coelomic fluid shines long and bright and decreases exponentially, by two orders of magnitude over two hours. Here, too, there are many nuances, depending on whether the coelomic fluid was freshly exuded or was already "exhausted". In the latter case, a new flash of light (decreasing in minutes) can be initiated by adding hydrogen peroxide or synthetic *Diplocardia longa* luciferin, which is a common substrate for luminous representatives of the megascolecoid families (Wampler & Jamieson 1980; see also Rota 2009; Rodionova *et al.* 2017) and Lumbricidae (in prep.).

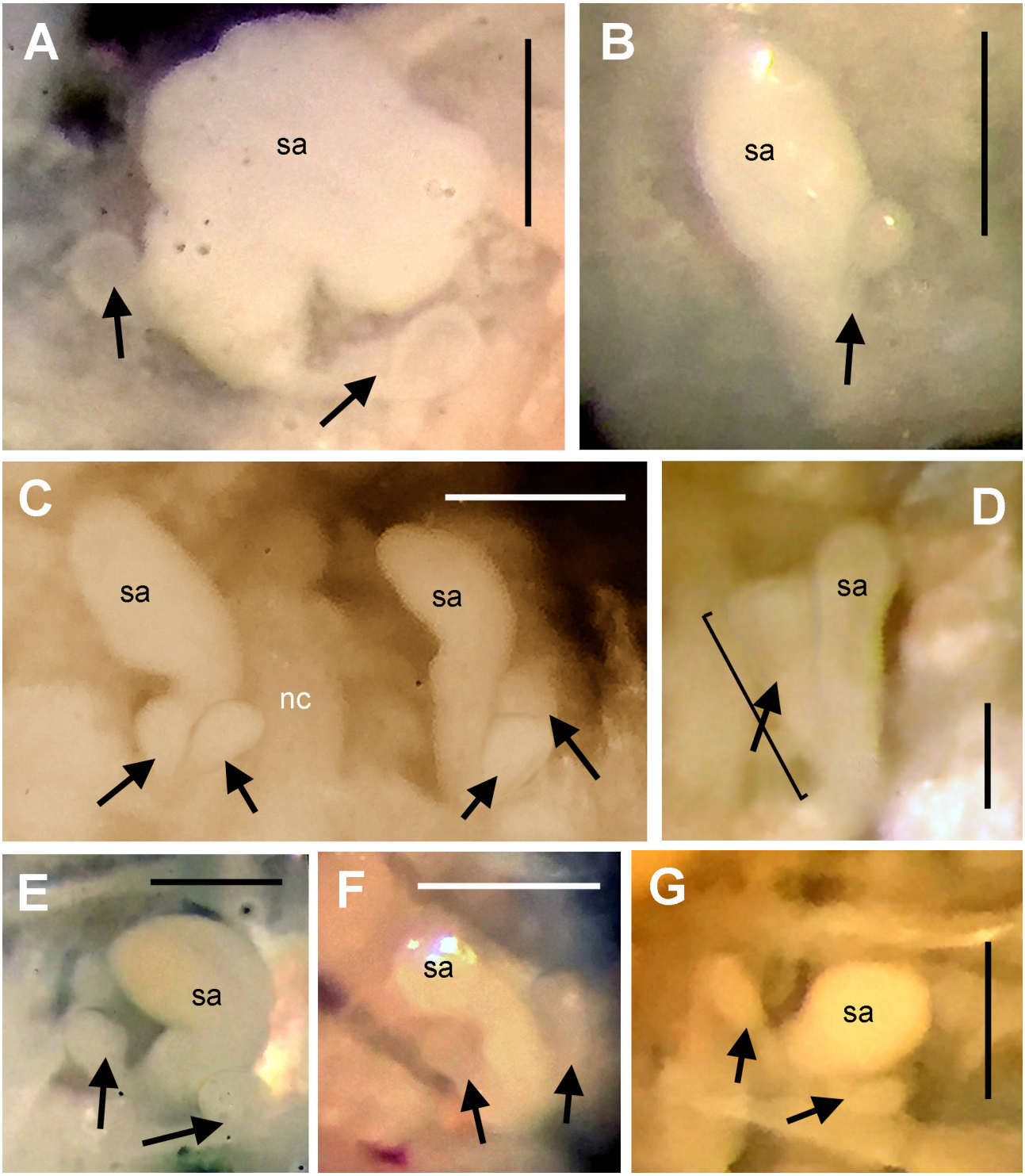

FIGURE 3. Close-up of the spermathecae (anterior view) on the floor of segment IX in specimens of various geographic origin, to show the respective size and shape of ampullae (sa) and diverticula (latter marked with arrows). **A.** Western Australia, CE16797. **B.** Western Australia, CE16798 (right spermatheca). **C.** Morocco, Mc124. Here both spermathecae are shown, on the two sides of nerve cord (nc), after removal of the alimentary canal and ventral vessel. **D.** Spain, Catalonia (left spermatheca; the square bracket indicates the length of the diverticulum). **E.** Italy, Sardinia, Guamaggiore. **F.** Italy, Apulia, Nardò. **G.** Italy, Sicily, Aeolian Islands, Vulcano. All scale bars = 200 µm.

Morphological comparisons. In our survey, we consistently measured the interchaetal distances at about segment XXVI and obtained the results shown in Table 1: the interval *ab* is the smallest and *dd* the largest in all material examined. The midlateral interval (*bc*) always exceeds (in most specimens by very little) the midventral

one (*aa*), except in one specimen from Catalonia. The ratio *bc*/*aa* had its highest values in worms from Morocco and Sicily. In the Siberian worms *aa* is smaller than *bc* both at segment IX and at segment XXVI; the dorsal distance *dd* behind the clitellum is only 1/6 of the body circumference (U), as compared to being 1/4–1/5 in the other worms examined. The ratio between *aa* and *cd* is variable, and in a single case (Sicily, Aeolian Is., Vulcano) the midventral interval (*aa*) was narrower than the intraspace of the upper chaetal couple (*cd*). The sequenced immature Spanish worm CE5290 from Valencia showed the same abnormal lessening of *ab* as compared to *dd* and to U as the specimens from Catalonia.

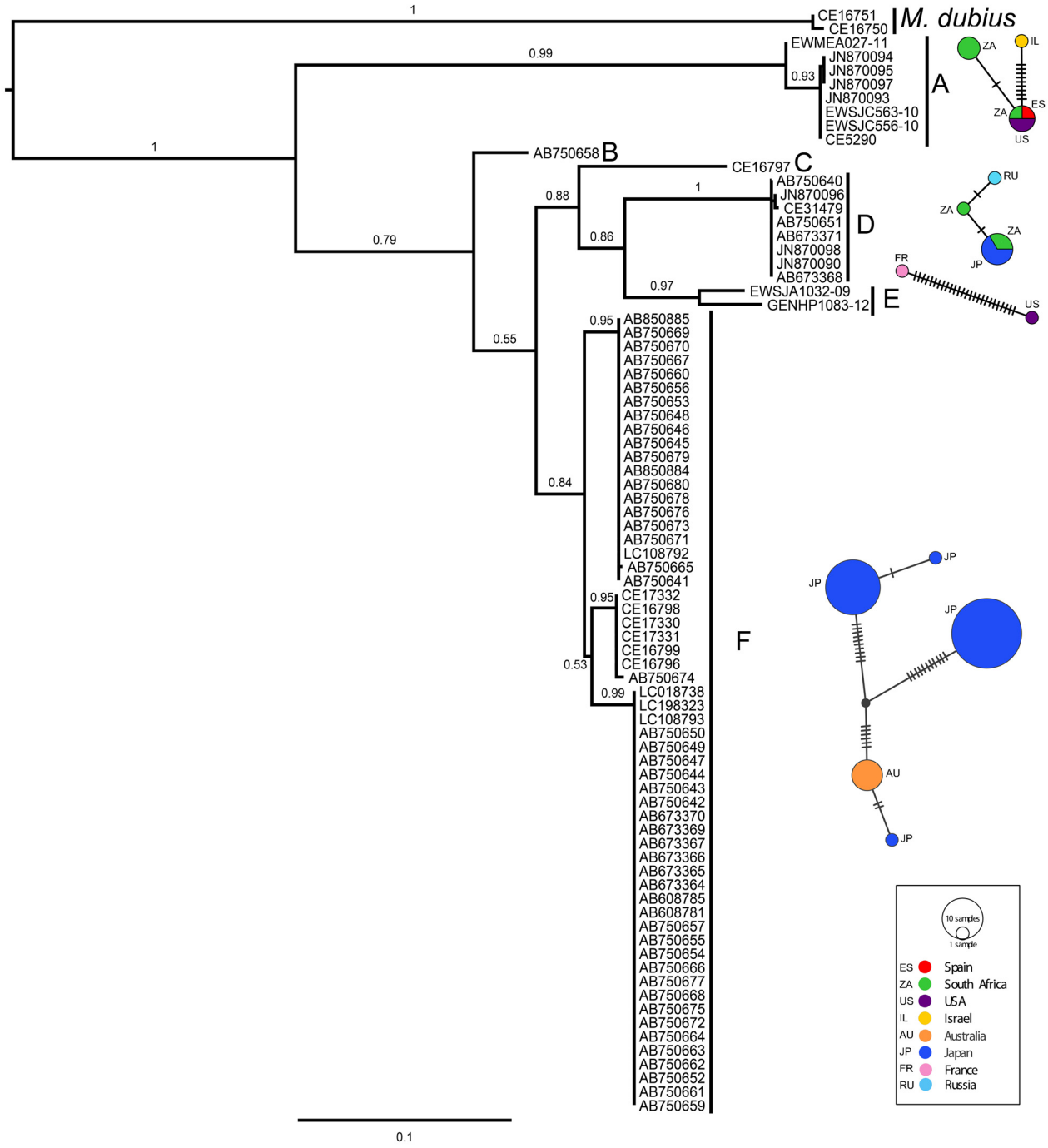

FIGURE 4. Gene tree and haplotype networks based on COI sequences illustrating the patterns of divergence of the sampled populations of *Microscolex phosphoreus* (Dugès, 1837) from Russia (Siberia), Australia and Spain as compared to specimens retrieved from GenBank and BOLD public libraries. In the networks, the colours together with two-letter country codes represent geographical origin. Hatch marks correspond to nucleotide substitutions.

TABLE 1. Sets of morphological features found useful to identify different morphotypes in *Microscolex phosphoreus* (Dugès, 1837). Columns from *aa* to *bc/cd*: Interchaetal features. Rest: Spermathecal features. In bold the key morphological characters distinguishing the individual morphotype. An asterisk followed by capital letter (A, C, D and F) marks the morphotypes considered in the molecular analysis and refers to the corresponding clade (see Figure 4 and Appendix 1).

	aa	*ab*	*bc*	*cd*	*dd*	U	U/*dd*	U/*aa*	*aa/cd*	*bc/aa*	*bc/cd*	Ampulla	# divert	shape divert.	loc. divert.
SIBERIA *D															
segm. IX, mean (*n* =3)	1.6	1	1.4	1.5	2.8	12.2	4.3	7.77	1.05	**0.89**	1.05				
segm. XXVI, mean (*n* = 5)	1.4	1	1.5	1.1	**1.7**	10.4	**6.2**	7.39	1.30	1.11	1.30	**round, lobed, large**	1	**small, knobstick-like**	medial/ant.
W AUSTRALIA *C															
CE16797, segm. XXVI	1.4	1	1.8	**1.3**	2.2	11.6	**5.2**	8.29	1.12	**1.25**	1.40	**round, lobed, large**	**2**	short, club-shaped	sides
W AUSTRALIA *F															
CE16796, 16798-99, segm. XXVI	1.5	1	1.7	1.1	2.4	11.3	4.7	7.49	1.44	1.09	1.56	long, club-shaped	1	**short, club-shaped**	**medial**
SPAIN *A															
CE5290, segm. XXVI	1.7	1	2.0	**1.3**	**3.0**	**13.4**	4.4	7.86	1.28	1.18	1.50	immature			
SPAIN															
Catalonia, segm. IX	**2.5**	1	**3.0**	1.5	**4.0**	**17.5**	4.3	7.00	1.67	1.20	2.00				
Same specimen, segm. XXVI	1.9	1	1.8	**1.3**	**3.2**	**13.3**	4.3	7.00	1.49	**0.95**	1.40	long, club-shaped	1	**long, club-shaped**	**medial**
MOROCCO															
Mc124, segm. XXVI	1.3	1	1.7	1.2	2.9	12.0	4.2	8.90	1.18	**1.30**	1.53	long, club-shaped	**2**	**short, club-shaped**	**anterior**
Mc125, segm. XXVI	1.2	1	1.7	1.2	2.5	11.5	4.6	9.16	1.09	**1.36**	1.48	long, club-shaped	1	short, club-shaped	side
ITALY, segm. XXVI															
Sicily, Vulcano Is.	1.5	1	**2.2**	**1.6**	2.8	**13.9**	4.9	9.27	**0.94**	**1.47**	1.38	long, club-shaped	2	short, club-shaped	sides
Sardinia, Guamaggiore	1.4	1	1.5	1.1	2.3	10.9	4.7	7.79	1.27	1.07	1.36	long, club-shaped	2	short, club-shaped	sides
Apulia, Gallipoli	1.5	1	1.9	1.3	2.9	12.8	4.4	8.53	1.15	1.27	1.46	long, club-shaped	2	short, club-shaped	sides
Apulia, Nardò-I	1.5	1	1.7	1.1	2.2	11.3	5.2	7.53	1.36	1.13	1.55	long, club-shaped	2	short, club-shaped	sides
Apulia, Nardò-II	1.2	1	1.5	1.1	2.1	10.5	5.0	8.75	1.09	1.25	1.36				

In all the investigated specimens the spermathecae have inconspicuous openings in 8/9 on line *a* and are located in IX. The variation observed in the morphology of the spermathecae within and between our samples examined is shown in Table 1 and Figures 2 and 3. The Siberian specimens have the largest ampulla, up to 410 µm wide; only the Western Australian CE16797 specimen has organs comparable in size (370 µm wide) (Fig. 3A). It is interesting to note that the three other specimens from that Australian sample represent a different morphotype (compare the spermathecae in Figures 3A and 3B, and the respective chaetal formulae in Table 1), and also differ by the COI haplotype (Appendix 1, Fig. 4). They share the main aspects of the spermathecal structure with the Spanish worms from Catalonia which however have a different chaetal formula.

Molecular variability. In the phylogenetic tree (Fig. 4), *M. phosphoreus* sensu lato is well separated from *M. dubius* and is divided into six main clades (A–F). Clade A, which is sister-group to the remaining *M. phosphoreus*, consists of specimens from Spain, Israel, USA and South Africa, followed by group B consisting of a single Japanese specimen. Groups C–E form a clade where group C consists of a single specimen from Australia, group D consists of specimens from Siberia, Japan, and South Africa, and group E consists of two specimens: one from USA and one from France. Finally group F, which is the largest clade, consists of 58 specimens from Australia and Japan. The minimum between-group genetic distances are greatest between *M. dubius* and the *M. phosphoreus* clades (0.180–0.194). Within *M. phosphoreus* the largest distances are found between group A and the other groups (0.146–0.156), and the smallest distance is between group B and F (0.063). The largest maximum within-group distances is found within group E (0.042) (see Table 2 for more details).

TABLE 2. Genetic distances (COI) for the sampled groups of *Microscolex phosphoreus* (A–F) and *M. dubius*. The distances for within-group comparisons are given as maximum pairwise distances, and for between-group comparisons as minimum pairwise distances. Within-group comparisons of groups consisting of singletons are not applicable (n/a).

	M. dubius	A	B	C	D	E	F
M. dubius	0.005						
A	0.194	0.016					
B	0.186	0.146	n/a				
C	0.186	0.151	0.091	n/a			
D	0.183	0.156	0.096	0.100	0.003		
E	0.190	0.156	0.091	0.089	0.086	0.042	
F	0.180	0.156	0.063	0.075	0.092	0.086	0.036

Discussion

The literature sources never indicate the precise segments at which the interchaetal distances were measured. We found the chaetal intervals at segment IX and XXVI to provide reliable markers of the respective haplotype groups. As concerns the variability in the spermathecal structure, we found good discriminating characters in the shape and size of the ampulla and in the position of diverticula, but not in their lobation or doubling. This agrees with Stephenson's (1914) and Omodeo's (1952) findings concerning intrapopulation and intraindividual variation. Gates (1972), who regarded *M. phosphoreus* as "a congeries of parthenogenetic morphs", justified the doubling of diverticula as part of a graded series of abnormalities that could be "read in either of two ways, fusing of two originally discrete diverticula, or splitting of an originally single diverticulum into two except for the common junction with the duct".

In their studies on the genetic diversity of *M. phosphoreus* in Japan, Oba *et al.* (2011–2016) identified five main haplotypes, three of which closely related (2.6–3.4%), two more divergent. Worms inhabiting a single Japanese locality could represent up to three such haplotypes, either close to (Nagoya University Higashiyama Campus), or divergent from each other (Hachijō-jima Island). Our inclusion of unidentified GenBank and BOLD sequences and the new sequences from Siberia, Australia and Spain showed that, even at global scale, there is no obvious geographic pattern in the data: most haplotype groups are found in several countries and specimens from the same countries are found in several groups. Specifically: our specimens from Western Australia mostly joined in the major Japanese clade (F), except for CE16797, which established a new, exclusive position (clade C). Our

Siberian specimen joined the clade containing Japanese and South African specimens (D), in a sister-group position to a clade formed by the Texas and French worms alone (E). Our specimen from Spain joined the remaining South African, Texas and Israel worms in the most external position (A), sister to all other *M. phosphoreus* (Fig. 4).

The combined morphological and genetic evidence suggest that at least clades A, C, D, and F should be reclassified as separate species, both morphologically and genetically distinct from each other (Table 1). As the number of specimens from which we have both genetic and morphological data is limited, and as only the COI gene has been studied for the genetic work, we hesitate in formally describing the species. There would also be the problem of assigning the available names to specific lineages. We do not have morphological data for the remaining two groups, B and E, and it is possible that they would fall in some of the other groups if more specimens and other markers were added. The only morphological description we have of a Japanese *M. phosphoreus* is that by Yamaguchi (1935), based on specimens collected on the sea-shore at Ôiso, Kanagawa. In them the spermathecae consisted each of a large main sac and a spherical diverticulum; the body circle (U) was about 14–18 times the chaetal distance *ab*; *ab*<*cd*<*bc*, *cd*<*aa*<*dd*; *bc*<=*aa* preclitellarly, *bc*>*aa* postclitellarly. Such chaetal features remind of those found by us in Spanish specimens (Table 1) and reported by Bouché (1972) for French specimens, which however all had club-shaped spermathecal ampullae and elongate diverticula.

Finally, Gates (1972) considered the possibility that *M. dubius*, which differs morphologically from *M. phosphoreus* sensu lato in having a joint male and prostatic pore on each side, in lacking spermathecae and in not being bioluminescent, could be a parthenogenetically degraded descendant of *M. phosphoreus.* Our study shows that the two synanthropic *Microscolex* are genetically well separate from each other. Moreover, our analyses revealed that several *M. phosphoreus* and *M. dubius* sequences in the public databases are misidentified, most often as members of the North American acanthodrilid genus *Diplocardia*. This could be avoided by examining the specimens' internal anatomy. For instance, *Diplocardia* species, unlike *M. dubius*, always have spermathecae, in numbers of three, two or (rarely) one pair (Gates 1977). The species *Diplocardia bitheca* Gates, 1977 may resemble *M. phosphoreus* in possessing only one pair of spermathecae and in lacking calciferous glands, but differs by having dorsal pores, two gizzards in V–VI, a typhlosole, avesiculate nephridia, nephropores in *d* (rather than in *c*), two pairs of prostates.

Habitat and bioluminescence of the Siberian specimens. From early January to mid May the entire surface of Lake Bajkal is covered in ice. The min and max temperatures at Bolshie Koty range from -25.5° and -15.5° C in January, to 12° and 24.5°C in July, respectively. The place is a rural settlement and in late spring/summer domestic greenhouses and the nearby fields are used for horticultural purposes, with heaters keeping the air warm overnight. The Bolshie Koty *M. phosphoreus* worms appear to be absent in the taiga soil nearby and most likely colonized the greenhouses from transplanted seedlings and plants. Greenhouses are unattended in wintertime, but nevertheless provide the Siberian population suitable conditions to survive the harsh local winter. In central Hungary the species tolerated outdoor winter temperatures of -20°C (Csuzdi 1986).

Microscolex phosphoreus belongs to the oligochaete family Acanthodrilidae where bioluminescence is most widely reported, occurring in the genera *Microscolex, Diplotrema, Diplocardia* (3 spp.), and *Parachilota* (Rota 2009). The bioluminescence system of *M. phosphoreus* was investigated with modern techniques by Wampler (1982), who also confirmed previous observations that luminescence comes from the disruption of granule-filled free coelomic cells discharged through the mouth and anus during stimulation (see Rota 2009). The general features of bioluminescence dynamics of the Siberian worms correspond to those already known, but the need by the Siberian specimens of a vigorous stimulation contrasts with the common knowledge of *M. phosphoreus* being an earthworm easily triggered to switch on, by stamping or by simply disturbing the soil in the vicinity (Stephenson 1930; Rota 2009). By comparison, even the bioluminescent Siberian enchytraeids *Fridericia heliota* and *Henlea* sp. are triggered by the slightest touch of the body or by simply hitting the soil (Rota *et al.* 2003). Also interestingly, the Siberian *M. phosphoreus* specimens produce *in vivo* a bright green bioluminescence (Fig. 1C), with a spectrum maximum at 530 nm, as compared to the maximum at 538 nm (Wampler 1982), perceived as yellowish green luminescence (Oba *et al*. 2011a), reported elsewhere for the species. Within *Diplocardia*, the different luminous species have emission spectra close but not identical to one another, with maxima at 500, 501 and 505 nm, respectively (Wampler & Jamieson 1980). The differences observed within *M. phosphoreus* concur to conclude that the taxon, as currently conceived, is a complex of more or less cryptic species.

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APPENDIX 1. List of *Microscolex* specimens used for the molecular study. Accession numbers in boldface are newly generated sequences, whereas the other sequences are from GenBank or BOLD, GenBank accession numbers have the prefix G, and BOLD accession numbers have the prefix B. The BIN codes refer to the Barcode Index Number system used by BOLD (where BINs are clusters of close barcode sequences that are assumed to correspond to species; Ratnasingham & Hebert 2013). Collection data and reference publication are given whenever available.

Microscolex dubius
BIN ABX5707; ID# CE16750; **G MH036527;** Australia, Western Australia, Dunsborough; 33°38'15.36"S; 115°7'1.20"E; C. Erséus & M.J. Wetzel; 16.9. 2012; (this study)
BIN ABX5707; ID# CE16751; **G MH036526;** Australia, Western Australia, Dunsborough; 33°38'15.36"S; 115°7'1.20"E; C. Erséus & M.J. Wetzel; 16.9. 2012; (this study)

Microscolex phosphoreus, Clade A
BIN AAM7540; ID# CE5290; **G MH036525;** Spain, Valencia; 39°27'10.04"N; 0°20'53.23"W; C. Erséus; 13.11.2008; (this study)
BIN AAM7540; B EWSJC556-10; USA, Texas
BIN AAM7540; B EWSJC563-10; USA, Texas
BIN AAM7540; G JN870093; South Africa; Voua Otomo *et al*. (2013)
BIN AAM7540; G JN870095; South Africa; Voua Otomo *et al*. (2013)
BIN AAM7540; G JN870097; South Africa; Voua Otomo *et al*. (2013)
BIN AAM7540; B EWMEA027-11; Israel

Microscolex phosphoreus, Clade B
BIN ACQ7593; G AB750658; Japan, Tokyo, Ota, Rokugodote; Oba *et al*. (2011a)

Microscolex phosphoreus, Clade C
ID# CE16797; **G MH036523;** Australia, Western Australia, Pemberton; 34°30'28.44"S; 116°5'18.24"E; C. Erséus, S. Martinsson, A. Pinder & Y. Cui; 20.9. 2012; (this study)

Microscolex phosphoreus, Clade D
BIN ACH5973; ID# CE31479; **G MH036524;** Russia, Siberia, Irkutsk; 51°54'23.5"N; 105°4'32.1"E; V.N. Petushkov, N.S. Rodionova (*via* E. Rota); 28.4. 2017; (this study)
BIN ACH5973; G AB673368; Japan, Shizuoka; Oba *et al*. (2011b)
BIN ACH5973; G AB673371; Japan, Shizuoka; Oba *et al*. (2011b)
BIN ACH5973; G AB750640; Japan, Shizuoka; Oba (2012)
BIN ACH5973; G AB750651; Japan, Tokyo, Hachijojima; Oba (2012)
BIN ACH5973; G JN870090; South Africa; Voua Otomo *et al*. (2013)
BIN ACH5973; G JN870096; South Africa; Voua Otomo *et al*. (2013)
BIN ACH5973; G JN870098; South Africa; Voua Otomo *et al*. (2013)

Microscolex phosphoreus, Clade E
AAL2102; B EWSJA1032-09; USA, Texas
ABX2182; B GENHP1083-12; France, Haute Normandie

Microscolex phosphoreus, Clade F
BIN ACQ6856; ID# CE16796; **G MH036517;** Australia, Western Australia, Pemberton; 34°30'28.44"S; 116°5'18.24"E; C. Erséus, S. Martinsson, A. Pinder & Y. Cui; 20.9. 2012; (this study)

BIN ACQ6856; ID# CE16798; **G MH036519;** Australia, Western Australia, Pemberton; 34°30'28.44"S; 116°5'18.24"E; C. Erséus, S. Martinsson, A. Pinder & Y. Cui; 20.9. 2012; (this study)
BIN ACQ6856; ID# CE16799; **G MH036522;** Australia, Western Australia, Pemberton; 34°30'28.44"S; 116°5'18.24"E; C. Erséus, S. Martinsson, A. Pinder & Y. Cui; 20.9. 2012; (this study)
BIN ACQ6856; ID# CE17330; **G MH036520;** Australia, Western Australia, Pemberton; 34°30'28.44"S; 116°5'18.24"E; C. Erséus, S. Martinsson, A. Pinder & Y. Cui; 20.9. 2012; (this study)
BIN ACQ6856; ID# CE17331; **G MH036521;** Australia, Western Australia, Pemberton; 34°30'28.44"S; 116°5'18.24"E; C. Erséus, S. Martinsson, A. Pinder & Y. Cui; 20.9. 2012; (this study)
BIN ACQ6856; ID# CE17332; **G MH036518;** Australia, Western Australia, Pemberton; 34°30'28.44"S; 116°5'18.24"E; C. Erséus, S. Martinsson, A. Pinder & Y. Cui; 20.9. 2012; (this study)
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