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MORPHOLOGICAL DIFFERENCES BETWEEN GENETIC LINEAGES OF THE PEREGRINE EARTHWORM *APORRECTODEA CALIGINOSA* (SAVIGNY, 1826)

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Aporrectodea caliginosa is a universally distributed and highly abundant peregrine earthworm that is the object of many ecological and ecotoxicological studies. Molecular phylogenetic analysis suggested that *A. caliginosa* consists of three highly diverged genetic lineages. In this study, we investigated morphological diversity within a sample of these three lineages from Belarus. We detected a variety of forms with different degrees of pigmentation and a shift in the clitellum position. The three genetic lineages of *A. caliginosa* demonstrated different propensity to particular morphological variants, including size, colour, and the clitellum position, yet no character could be used to distinguish among the lineages with sufficient accuracy. Thus, our results suggest that identification of the genetic lineage should be recommended for ecological studies involving *A. caliginosa* to account for possible differences between them.

Key words: *Aporrectodea caliginosa*, earthworms, Lumbricidae, morphological variation, genetic lineages, cryptic diversity.

INTRODUCTION

Aporrectodea caliginosa (Savigny, 1826) is an endogeic peregrine earthworm. Its original distribution (before the Last Glacial Maximum) was probably in southern Western Europe (Hendrix *et al.* 2008), from where it spread to all continents except Antarctica (Tiunov *et al.* 2006, Porco *et al.* 2013, Bart *et al.* 2018). *A. caliginosa* is one of the most abundant earthworms found in many natural and particularly agricultural land types in the temperate zone (Boag *et al.* 1997, Vsevolodova-Perel 1997, Ivask *et al.* 2007).

A. caliginosa is a part of a complex of closely related species, which also includes *A. trapezoides*, *A. nocturna*, and *A. tuberculata*, as well as several other species; the list of the included taxa and their status varies according to different authors (Sims & Gerard 1985, Pérez-Losada *et al.* 2009, Fernández *et al.* 2012). Morphological delimitation among the members of the complex is vague. Molecular analyses recovered *A. caliginosa* either as monophyletic (Pérez-Losada *et al.* 2009, Fernández *et al.* 2012) or as polyphyletic (Latif *et al.* 2020); this issue requires a multigene nuclear dataset to be satisfactorily resolved.

The presence of highly diverged genetic lineages is a well-known phenomenon among earthworms (King *et al.* 2008, Novo *et al.* 2009, Decaëns *et al.* 2013, Marchán *et al.* 2018, Shekhovtsov *et al.* 2019). Three genetic lineages of *A. caliginosa* have been reported (Porco *et al.* 2013). Although genetic lineages of earthworms are generally considered cryptic, sometimes significant morphological diversity can be found in certain populations (Shekhovtsov *et al.* 2016). It is unclear if certain genetic lineages are truly cryptic or whether there are minor morphological differences among them that have been overlooked (Marchán *et al.* 2020).

In this study, we investigated the morphological diversity in a sample of the three *A. caliginosa* genetic lineages from Belarus. We documented various morphological variations that slightly deviate from the typical diagnosis, as well as cases where an individual could be identified as a different species. All specimens were genotyped to verify the morphological identification. Our aim was (1) to identify possible morphological characters that vary between genetic lineages of *A. caliginosa* and (2) to document the extent of morphological variation of the species compared to published diagnoses and the extent of overlap with other species.

MATERIALS AND METHODS

A. caliginosa individuals were collected in 2019 in Belarus (Fig. 1) and fixed in ethanol. GPS coordinates of the sampled locations are as follows: location 1, 55.6419°N, 27.0408°E; loc. 2, 54.9716°N, 26.8700°E; loc. 3, 54.9674°N, 26.8704°E; loc. 4, 54.2142°N, 25.9661°E; loc. 5, 53.2082°N, 26.1010°E; loc. 6, 52.0673°N, 24.9429°E; loc. 7, 54.7315°N, 28.1247°E; loc. 8, 55.7302°N, 27.7765°E; loc. 9, 55.9107°N, 27.8889°E; loc. 10, 56.1365°N, 28.1378°E; loc. 11, 55.1735°N, 28.8881°E; loc. 12, 55.0518°N, 29.7150°E; loc. 13, 54.4241°N, 29.7967°E; loc. 14, 55.1713°N, 30.2665°E; loc. 15, 54.5202°N, 30.2775°E; loc. 16, 54.5175°N, 30.2975°E.

Preliminary morphological identification was performed according to Vsevolodova-Perel (1997). Individuals slightly deviating from the typical diagnosis (Table 1) were taken for the subsequent analysis. We assessed their size (length, width, the length and width of the clitellum), the number of segments, colour, and pigmentation. Welch's test estimated morphological differences between the lineages. Length and width were measured with an

accuracy of 0.5 mm. For statistical analysis, the position of the clitellum was encoded with an accuracy of 0.25× segments.

Sequencing of the fragment of the cytochrome c oxidase I gene (COI) was performed with the universal primers LCOm (5′-TACTC-AACAA-ATCAC-AAAGA-TATTG-G-3′; modified from Folmer *et al.* 1994) and COI-E-(5′-TATAC-TTCTG-GGTGT-CCGAA-GAATC-A-3′; Bely & Wray 2004) with the Biomaster HS-Taq PCR Mix (Biolabmix, Russia) as described in Shekhovtsov *et al.* (2018*a*). The amplified fragments were assessed using agarose gel electrophoresis and cleaned by the shrimp alkaline phosphatase/*E. coli* exonuclease I mix (New England Biolabs, USA). Sanger sequencing was conducted on a 3130xl DNA Analyzer (Applied Biosystems) in SB RAS Genomics Core Facility (ICBFM SB RAS, Novosibirsk, Russia) using both forward and reverse primers. The obtained DNA sequences were manually edited and assembled using Chromas Lite v. 2.0 (www.technelysium.com.au/).

Species/lineage identification was performed using Blast (blast.ncbi.nlm.nih.gov/Blast.cgi) according to the published sequences of *A. caliginosa* from the studies of Pérez-Losada *et al.* (2009), Fernández *et al.* (2012), Porco *et al.* (2013), Shekhovtsov *et al.* (2016, 2018*b*). Only unique sequences that were not yet present in GenBank were submitted under accession numbers MW080717–MW080729. Phylogenetic trees were built using the Maximum Parsimony and Maximum Likelihood algorithms with the Mega X program (Kumar *et al.* 2018). Maximum Parsimony trees were built using the subtree-pruning-regrafting search algorithm. Maximum Likelihood algorithm used the General Time Reversible (GTR+I+G) model; 1000 bootstrap replicates were performed for each algorithm.

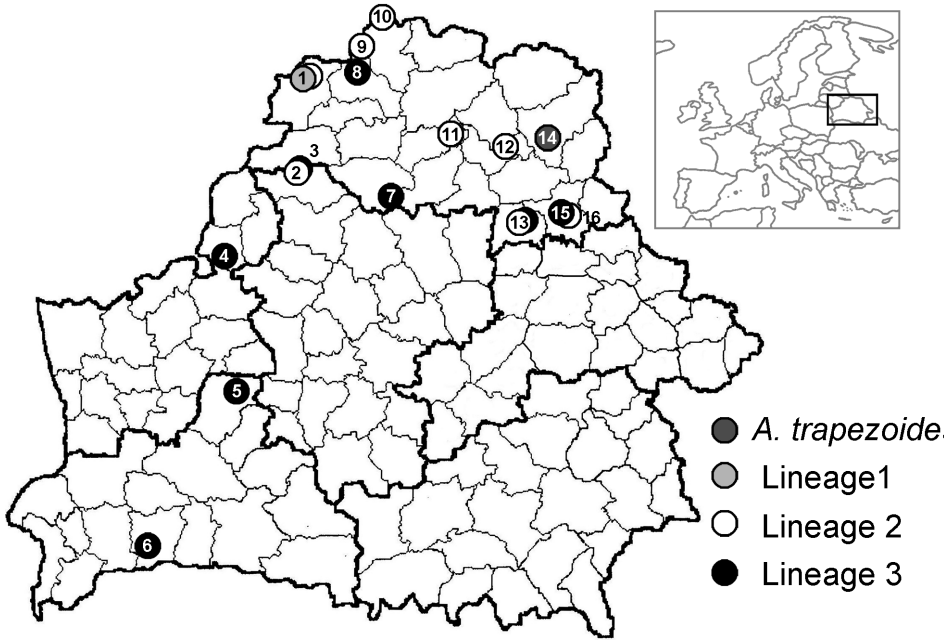

Fig. 1. Sampled sites of *A. caliginosa*

Table 1. Morphometry of the *A. caliginosa* individuals studied here. Nseg = number of segments, L = body length in mm, W = body width in mm, CS = clitellum start, CE = clitellum end, CL = clitellum length in mm, CW = clitellum width in mm, TP = *tuberculae pubertatis*, '' = ditto, cli. = clitellum. Haplotype refers to GenBank accessions.

Pigmentation	Nseg	L	W	CS	CE	CL	CW	TP	haplotype
				Lineage 1					
absent	92	41	2	28	34.25	3	3		MW080725
''	121	74	2.5	28.75	34.25	4	3.5		''
''	111	55	2.5	28	34	3.5	3		MW080722
''	108	46	2	28	34.25	3.5	3		MW080723
''	106	52	2	28.5	34.25	3.5	3		MW080724
''	114	51	2	28.75	34.25	3.5	3		MW080728
yellowish, post-cli.	120	36	2	28.75	34.25	3.5	3		MW080729
Average	110.3	50.7	2.1	28.4	34.2	3.5	3.1		
				Lineage 2					
absent	157	62	4	27	35	4.5	4.5		KU358811
''	158	68	4	27	35	5	5		''
''	150	81	4	27.5	35	5.5	5		KF471807
reddish, pre-cli.	144	59	3	29	34.25	4	3.5		KU358811
''	111	47	3	27.75	34.25	4	4		KU358777
''	177	69	4	27.75	34	4	5		KU358820
''	130	78	3.5	28	34	5	4		''
''	141	67	3.5	28	34.25	5	4		''
''	149	67	3.5	28	34.25	5	4		''
''	161	71	4	28	34.25	5	4.5		MW080718
''	170	61	3.5	27.75	34	4	4		KU358757
''	155	73	3.5	27.75	34	5	4		KU358747
''	139	56	3.5	28	35	4	4		''
''	167	71	3.5	27.5	34	5.5	4		
reddish, post-cli.	173	83	5	27	34.25	5	5.5		KF471807
brown, pre-cli.	155	61	3	28	34.25	4	4		KU358826
''	167	69	3	28	35	4	4		MW080717
brown, post-cli.	164	73	4	28	34.25	5	5		KF471794
''	151	62	4	27	34	4	5		KF471807
''	128	59	5	28	34	4	5		KF471794
''	160	58	4	28	34.25	4	4.5		''
Average	152.7	66.4	3.7	27.8	34.3	4.5	4.4		

Table 1 (continued)

Pigmentation	Nseg	L	W	CS	CE	CL	CW	TP	haplotype
				Lineage 3					
absent	133	51	2.5	28	34	4	3		KU358858
‘’	123	65	3	28.75	34.25	4	3.5		‘’
‘’	130	46	2.5	29	35	3	3	32–34	‘’
‘’	133	55	3	27.75	34	4	4		‘’
‘’	138	54	3	29	34.25	4	3.5		‘’
‘’	125	55	3	28	34	4.5	3.5		‘’
‘’	130	50	2.5	28	34	4	3		‘’
‘’	130	42	2	29	34	3	3		‘’
‘’	132	38	3	27	37	3.5	3.5	30–32	‘’
‘’	114	69	3	28.75	34	4.5	4	as bands	MW080721
‘’	126	66	3	28.75	34.25	5	4		KU358856
‘’	131	71	3	28.75	34.25	4.5	4		KU358841
‘’	158	58	3	27.5	34.5	4	3.5		KU358862
‘’	156	77	3	28.5	34.5	5	4	as bands	MW080727
‘’	134	39	2.5	29	34	3	3		MW080726
reddish orange, pre-cli.	163	53	3	28.75	34.25	3.5	4		KU358862
‘’	147	46	3	28.75	34	4	4		‘’
‘’	147	47	2.5	28.75	34	4	3		KU358858
‘’	150	49	3	28.75	34	4	4		KU358873
yellowish, pre-cli.	131	71	3.5	28.5	34.5	4	4		KU358858
‘’	137	52	3	28.75	34.25	4	3.5		‘’
‘’	139	52	3	28.5	35	4.5	4		KU358862
‘’	118	51	3	28	34.25	4	4		‘’
‘’	166	56	3	28.5	34.5	4	4		‘’
‘’	144	55	3	28.5	34.5	4.5	4		‘’
‘’	143	47	3	28.75	34	3.5	4		‘’
‘’	136	46	3	28.75	34	3	4		‘’
yellowish, post-cli.	131	78	3	28.5	34.5	5	4		MW080719
greyish brown, post-cli.	142	68	3.5	28	34.25	4	4		MW080720
brown, post-cli.	124	46	2	28	35	3.5	3		KU358862
Average	137.0	55.1	2.9	28.5	34.4	4.0	3.7		
				A. trapezoides					
brown, post-cli.	165	80	4	27	35	5.5	5		KT073944

RESULTS

We studied a total of 251 adult *A. caliginosa* individuals from Belarus. In this sample, we identified a set of 59 individuals (Table 1) with deviations from the diagnosis of Vsevolodova-Perel (1997). The final sample included earthworms with different degree of pigmentation: reddish-brown, yellowish-brown or light red, as well as different variants of the position of the clitellum. The position and form of the *tuberculae pubertatis* were typical for *A. caliginosa*, except for four individuals.

DNA sequencing demonstrated that the studied sample contained all three known lineages of *A. caliginosa*. The extent of genetic differences between the lineages was similar to that found in previous studies (Fig. 2). Differences in most parameters among the lineages turned out to be statistically

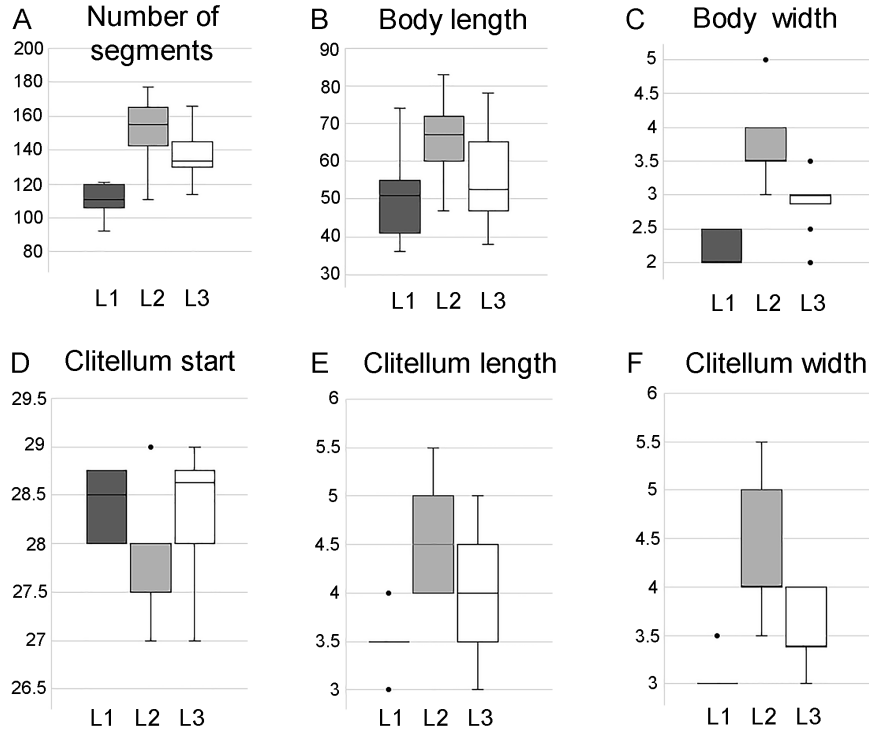

Fig. 2. Box with whiskers plots for the data in Table 1: A, number of segments; B, body length (mm); C, body width (mm), D, clitellum start; E, clitellum length (mm); F, clitellum width (mm). Dark grey, lineage 1; light grey, lineage 2; white, lineage 3. Boundaries of the box stand for the 25th and the 75th percentiles; line within the box denotes the median; if the median coincides with box border, it is shown as a bold line; whiskers represent the 1.5 interquartile ranges; dots stand for outliers.

Table 2. Comparison among *A. caliginosa* lineages using Welch's t-test; L1, L2, L3 refer to the corresponding genetic lineages.

	L1 vs. L2	L1 vs. L3	L2 vs. L3
Body length	<0.05	–	<0.001
Body width	<0.001	<0.001	<0.001
No. of segments	<0.001	<0.001	<0.001
Clitellum start	<0.01	–	<0.001
Clitellum end	–	–	–
Clitellum length	<0.001	<0.01	<0.01
Clitellum width	<0.001	<0.001	<0.001

significant: body length and width, clitellum length and width, the number of segments and the anterior position of the clitellum (Table 2). Lineages 1 and 3 were closer in these parameters to each other than to lineage 2. The only parameter that demonstrated no differences among the lineages was the posterior position of the clitellum.

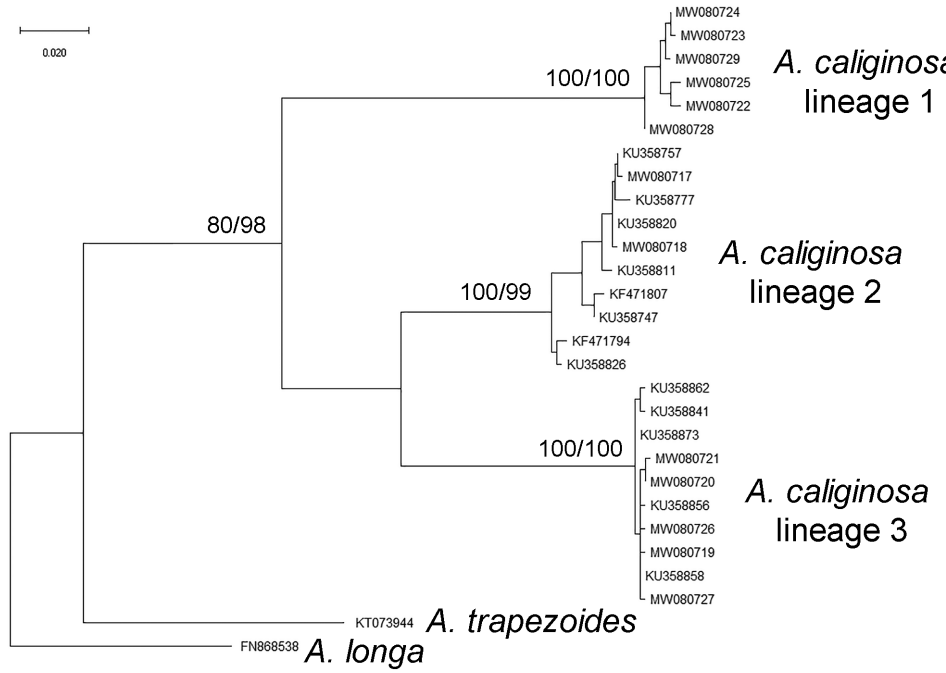

Fig. 3. Maximum likelihood phylogenetic tree of the obtained sequences. Numbers near the branches denote Maximum Parsimony/Maximum Likelihood bootstrap support

DISCUSSION

Morphological variation in A. caliginosa

Earthworms are generally identified using identification keys and diagnoses. However, published diagnoses tend to set strict limits and omit outlier variants that may account for a significant portion of the population. Moreover, the descriptions of *A. caliginosa* vary in different sources. In the former Soviet Union, *A. caliginosa* is usually identified using Vsevolodova-Perel's (1997) key, which specifies the position of the clitellum on the segments 27 – 34/35. Other sources give a broader diagnosis, e.g. 25/26/(29) – 34/35 (Csuzdi & Zicsi 2003, Mezhzherin *et al.* 2018). In the sample from Belarus, clitellum started on the segments 27/28/29 and ended on 33 or 34. Earlier we reported a population of *A. caliginosa* from Russia with the clitellum ranging from 26th to the 32nd segments (Shekhovtsov *et al.* 2016). Thus, the clitellum position in *A. caliginosa* may range from 25/26/27/28/29 to 32/33/34/35. This variation may partly be explained by the fact that the clitellum's extent varies depending on the reproductive cycle: during the maximum extent of clitellum it encroaches on the neighbouring segments (our observations).

The *tuberculae pubertatis* turned out to be a more constant character. The shift in their position was observed in only two specimens, from the segments 31–33 to 30–32 and 32–34, respectively. Form of *tuberculae pubertatis* is an important character that distinguishes *A. caliginosa* from the closely related *A. trapezoides* (alongside with pigmentation). The majority of the studied individuals had *tuberculae pubertatis* as two more or less pronounced protuberances, typical for *A. caliginosa*. However, in two individuals, those formed pads, characteristic for *A. trapezoides*.

Body colour is an important character in the *A. caliginosa* complex: *A. caliginosa* differs from the closely related *A. trapezoides* by the absence of pigmentation. Although most of the studied individuals were nonpigmented, some had reddish, brownish, or yellowish pigmentation, thus resembling *A. trapezoides* or *A. nocturna*. Similar variation in colouration was reported in Ukraine (Mezhzherin *et al.* 2018). DNA analysis identified most of the specimens studied by us as *A. caliginosa*. However, an individual from one location turned out to be the real *A. trapezoides*, representing the first reported case of this species for Belarus (Maksimova & Gurina 2014).

As seen from the data above, morphological variation in *A. caliginosa* is significant, and based on the external characters some of the pigmented individuals could be identified as *A. trapezoides* or *A. nocturna*, depending on the key used. *A. trapezoides* differs from *A. caliginosa* by having brown pigmentation and by the form of *tuberculae pubertatis* (pads instead of protuberances; Vsevolodova-Perel 1997). *A. nocturna* is a large endogeic earthworm

differing from *A. caliginosa* by its size and dark brown pigmentation (Sims & Gerard 1985); however, size can also vary widely and some individuals in our sample were close to the lower size limit specified for *A. nocturna*. Moreover, the individual with the *tuberculae pubertatis* located on the segments 30–32 was morphologically closer to *Eisenia uralensis* Malevič, 1950, an endemic species from the Urals (Vsevolodova-Perel 1997). Thus, we could conclude that the accuracy of identification and the resulting species count can be heavily influenced by intraspecific variation, and DNA analysis is required to clarify contentious cases.

Differences between genetic lineages

Different lineages demonstrated distinct tendencies towards particular character states when comparing the sample of individuals with slightly aberrant phenotypes. Lineage 1 was significantly smaller than lineages 2 and 3, with fewer segments and smaller clitellum (Table 1, Fig. 2); only one pigmented individual was detected for this lineage. Lineage 2 was the biggest of all three and also tended towards earlier start of the clitellum, by about ¾ of a segment compared to lineage 3.

We should note that the sample was collected from multiple locations, and such characteristics as size and colour may be affected by environmental factors (Piearce *et al.* 2002; García & Fragoso 2003), so controlled laboratory experiments are needed to verify these morphological differences. On the other hand, genetic differences between the lineages might make them occupy somewhat different environments.

A. caliginosa is recognized as one of the most important and widespread earthworms associated with agricultural lands (Boag *et al.* 1997, Vsevolodova-Perel 1997, Ivask *et al.* 2007) and thus as an important model organism in ecological and applied studies (Bart *et al.* 2018). However, as seen from our results, different genetic lineages demonstrate significant differences and thus may behave differently in such studies. It thus seems that identification of the genetic lineage used should be recommended for ecological studies involving *A. caliginosa* to account for possible biases when comparing different studies.

CONCLUSIONS

In this study, we investigated morphological diversity within *A. caliginosa* and detected a variety of forms with different degrees of pigmentation and shifts in the position of the clitellum; some of the studied individuals could be identified as representatives of other species based on the external morphology alone. The three genetic lineages of *A. caliginosa* demonstrated

a different propensity to particular morphological variants, yet no character could be used to distinguish among the lineages with sufficient accuracy. This indicates that these lineages may have different ecological characteristics that should be taken into account in ecological and ecotoxicological studies.

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