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The *Allolobophora sturanyi* species group revisited: Integrated taxonomy and new taxa (Clitellata: Megadrili)

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Abstract. The *Allolobophora sturanyi* Rosa, 1895 species group is revisited using DNA barcoding and morphology. Barcoding results corroborated the previous treatment of the *Allolobophora sturanyi* subspecies and furthermore proved that the morphologically similar *Allolobophora gestroides* Zicsi, 1970 species belong to this species group. Elaboration of new samples from the Apuseni Mts resulted in discovery of a new subspecies *A. sturanyi biharica* ssp. nov. from the summit of the Bihor range, and a new species *A. zicsica* from the Vladeasa range similar to *A. gestroides* described from Northern Hungary.

Keywords. Earthworms, barcoding, COI, Carpathian Basin, new species

INTRODUCTION

The *Allolobophora sturanyi* species group was first recognized by Csuzdi & Pop (2008) through morphometric and biogeographic analysis of the species distributed in the Balkan and the Carpathian Basin and previously thought to be related to the Franco-Iberian *Allolobophora dugesi* (Rosa, 1886). The non-metric multidimensional scaling (MDS) and cluster analysis (CA) resulted in four well separated groups which showed distinct biogeographical patterns as well (Csuzdi & Pop 2008). On the basis of the somewhat variable positions of the clitellum and tubercles, and furthermore the varying number of spermathecae, three lineages were treated as *Allolobophora sturanyi* subspecies, namely *A. s. sturanyi* Rosa, 1895, *A. s. dacica* (Pop, 1938) and *A. s. dacidoides* Bouché, 1973. The fourth clade differing markedly in the position of the clitellum from *A. sturanyi,* was described as a new species *Allolobophora prosellodacica* Csuzdi & Pop, 2008.

In the recent years new samples were collected in the Apuseni Mts and the Carpathians including a specimen, in habitus resembling the *A. sturanyi* subspecies, but its clitellum begins much backwards, on segment 32 and therefore taxonomically similar to *Allolobophora gestroides* Zicsi, 1970 which was described from Northern Hungary.

Another sample, collected on and around the highest peak of the Bihor Mts (Cucurbăta Mare) contained also a strange species seemingly similar to *A. sturanyi* but it was not identical to any of the previously described subspecies.

To clear the positions of the newly discovered specimens and the species/subspecies in the *A. sturanyi* species group, in addition to the morphological investigation, we have carried out a molecular phylogenetic analysis using barcode sequences (COI) from all *A. sturanyi* subspecies and also *A. gestroides*. Unfortunately we were not able to collect fresh material from *A. prosellodacica* suitable for DNA extraction.

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MATERIALS AND METHODS

Earthworms were collected by the diluted formaldehyde method (Raw 1959) supplemented with digging and hand-sorting and also looking under stones and fallen logs. The worms were killed in 96% ethanol and preserved in 75% ethanol. For molecular studies, some specimens were placed into 96% ethanol. The identified material is deposited in the Soil Zoology Collection of the Hungarian Natural History Museum (HNHM).

DNA extraction, amplification and sequencing were carried out in the Molecular Taxonomic Laboratory of HNHM according to the protocol described by Szederjesi & Csuzdi (2015). In addition to the newly got barcodes several sequences were downloaded from the GenBank (Online Appendix 1).

DNA sequences were aligned with ClustalW implemented in MEGA 6.06 (Tamura *et al*. 2013) using the default settings.

Maximum Likelihood (ML) analysis was carried out using the online tool on Phylogeny.fr (Dereeper *et al.* 2008) with GTR G+I substitution model selected by the model selection process implemented in MEGA 6.06 (Tamura *et al.* 2013) and 100 bootstrap replication.

Bayesian inference was performed using the BEAST 1.8.2 software (Drummond *et al.* 2012) with the best fitting GTR G+I substitution model. The analysis was run for 10 millions of generations, sampling trees at every 1000th generation. The first 2000 trees were discarded as "burn in" in TreeAnnotator v.1.8.2. The resulted tree was visualized with FigTree 1.4.2 (Rambaut 2014).

RESULTS

The *A. sturanyi* species group proved to be monophyletic in both (Bayesian and ML) analyses (Fig. 1) however, with moderate support (79% and 61% respectively). The new species *A. zicsica* sp. nov. forms a quite well supported clade (88% and 87%) with *A. gestroides* and this clade is basal to the *A. sturanyi* subspecies. The relationships between the *A. sturanyi* subspecies are not quite clear and the Bayesian and ML analyses resulted in two different topologies. In the ML tree *A. s. sturanyi* is basal to the other subspecies and *A.* s. *biharica* is close to *A. s. dacica* however, in the Bayesian tree (Fig. 1B) *A. s. biharica* forms a moderately supported clade with *A. s. dacidoides*. The morphological characters support this latter hypothesis, because with 2/3 pairs of spermathecae *A. s. biharica* from the summit of the Bihor Mts resembles *A. s. dacidoides* distributed in higher elevations of the Carpathians and characterized by 3/4 pairs of spermathecae (Table 2).

According to the thorough analysis by Chang & James (2011) the K2P distances lower than 9% and higher than 15% can unambiguously be assigned to the same species or two different species, respectively. Between these two values there is an ambiguous range requires further considerations. The K2P genetic distances in the *A. sturanyi* species group (Table 1) vary between 10.6% and 14.1% which corroborate the previous taxonomic conclusion of Csuzdi & Pop (2008) that *dacica*, *dacidoides* and also *biharica* ssp. nov. represent different subspecies of *A. sturanyi*.

The genetic distance (K2P) between *A. gestroides* and *A. zicsica* is 16.1% which proves their different specific statuses on molecular level too. It is worth noting that the genetic distance between *A. gestroides* and *A. s. dacidoides* is 14.6% however, the large morphological differences (Table 2) verify that they represent different species.

Table 1. K2P genetic distances between the *A. sturanyi* species group taxa. Intraspecific distances are in bold.

	1	*2*	*3*	*4*	*5*
A. s. dacica	***0.064***				
A. s. dacidoides	*0.141*	***0.076***			
A. s. biharica	*0.124*	*0.106*	-		
A. s. sturanyi	*0.137*	*0.139*	*0.115*	-	
A. gestroides	*0.169*	*0.146*	*0.157*	*0.171*	-
A. zicsica	*0.179*	*0.176*	*0.169*	*0.182*	*0.161*

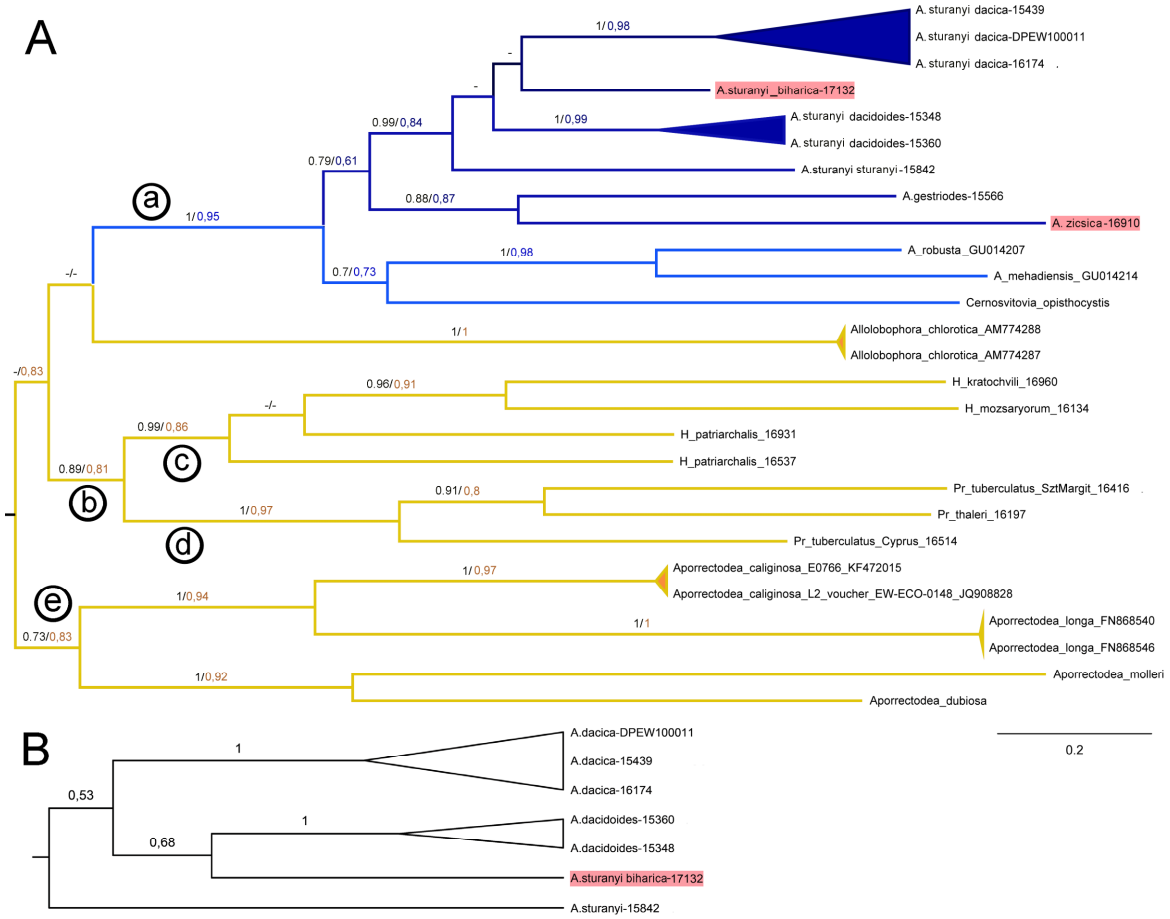

Figure 1. Phylogenetic reconstruction of the *A. sturanyi* species group (dark blue). Yellow clades represent outgroup species. Numbers above clades are Bayesian posterior probabilities/ bootstrap supports. A = ML tree, B = Bayesian tree.

TAXONOMY

Allolobophora zicsica Szederjesi, Pop & Csuzdi sp. nov.

(Figures 2–3)

Material examined. Holotype. HNHM/16910, Romania, Săcuieu, Vf. Bogdanului, 46.81°N 22.92°E, mixed pine-beech-oak forest, leg. J. Novák, T. Szederjesi, 19.04.2014.

Diagnosis. Length 45 mm, diameter 3.5 mm, setae closely paired. Pigmentation lacking. First dorsal pore on 8/9. Clitellum on 32–40, tubercles on ½35–½40. Male pore on 15, large (Fig. 2). Nephridial pores invisible. Two pairs of vesicles in 11, 12; spermathecae three pairs in 9/10–11/12 in *cd*. Calciferous glands with well-developed diverticula in 10. Hearts in segments 6–11, nephridial bladders proclinate, J-shaped.

External characters. Holotype 45 mm long and 3.5 mm wide. Number of segments 125. Colour pale, pigmentation lacking. Prostomium epilobous ½ closed. First dorsal pore at intersegmental furrow 8/9. Setae closely paired. Setal arrangement behind clitellum: *aa*:*ab*:*bc*:*cd*:*dd* = 16.3:1.3:6.3:1:23.6 (Fig. 3). Male pores on segment 15, surrounded by glandular crescents. Nephridial pores invisible. Clitellum on segments 32–40. Tubercula pubertatis on segments ½35–½40. Genital papillae on segments 13, 14, 16, 29, 30, 36–40 *ab*.

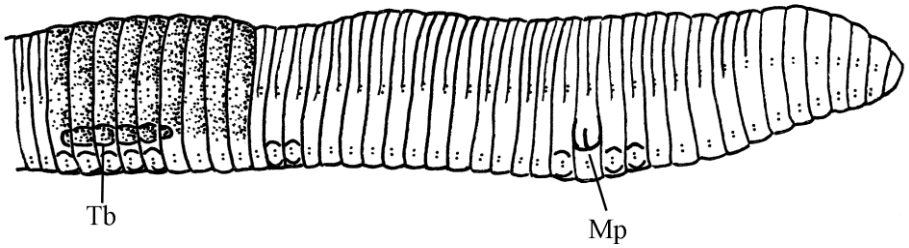

Figure 2. *A. zicsica* sp. nov. Ventrolateral view of the clitellar region. Mp = male pore, Tb = tubercle.

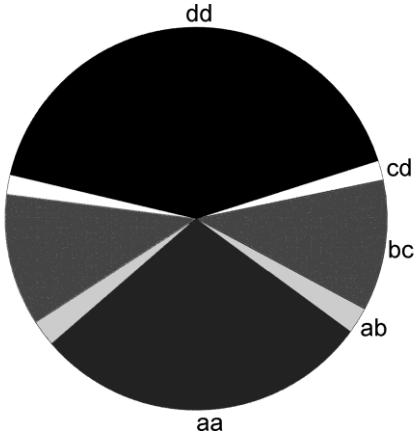

Figure 3. *A. zicsica* sp. nov. Setal arrangement.

Internal characters. Dissepiments 5/6–8/9 strongly thickened, 9/10 slightly thickened. Testes and funnels in segments 10–11, covered by perioesophageal testis sacs. Seminal vesicles in 11, 12. Spermathecae three pairs in 9/10, 10/11, 11/12 with external openings near the setal line *cd*. Calciferous glands with well-developed diverticula in segment 10. Paired hearts appear in segments 6–11, with a pair of small extraoesophageal vessels in 12. Nephridial bladders J-shaped with short proclinate ental part. Crop in segments 15–16, and gizzard in segments 17–18. Typhlosolis trifid, longitudinal musculature is of fasciculated type.

Etymology. The new species is named in honour of the late Prof. Dr. András Zicsi, the renowned earthworm taxonomist.

Remarks. *A. zicsica* sp. nov. is close to *A. gestroides* but differs in the position of the clitellum (32–40 *vs.* 29, 30–40,41), smaller biometry (45 x 3.5 mm *vs.* 55–98 x 4.3–5.2 mm) and 16.1% K2P genetic distance.

Allolobophora sturanyi biharica Szederjesi, Pop & Csuzdi ssp. nov.

Figures (4–5)

Material examined. Holotype. HNHM/17133, Romania, Bihor Mts., before the peak of Curcubăta Mare, 1810–1685 m, 46°26.126'N 22° 42.761'E, leg. Csuzdi Cs., 19.06.2015. *Paratypes*. HNHM/17151, 8 ex., locality and date same as of Holotype.

Diagnosis. Length 79–91 mm, diameter 3–3.5 mm, setae closely paired. Pigmentation lacking. First dorsal pore on 9/10. Clitellum on 27–38, tubercles on 28–½37. Male pore on 15, small (Fig. 4). Nephridial pores irregularly alternate between setal line *b–d*. Two pairs of vesicles in 11, 12; spermathecae two or three pairs in 9/10–10/11(11/12) in *cd*. Calciferous glands with well-developed diverticula in 10. Hearts in segments 6–11, nephridial bladders proclinate, J-shaped.

External characters. Holotype. 84 mm long and 3 mm wide. Number of segments 205. *Paratypes*. 79–91 mm long and 3–3.5 mm wide, number of segments 179–182. Colour pale, pigmentation lacking. Prostomium epilobous 1/3 closed. First dorsal pore at intersegmental furrow 9/10. Setae closely paired. Setal arrangement behind clitellum: *aa*:*ab*:*bc*:*cd*:*dd* = 24:1.5:13:1:48 (Fig. 5). Male pores on segment 15, small. Nephridial pores irregularly alternate between setal line *b–d*. Clitellum on segments 27–38. Tubercula pubertatis on segments 28–½37. Genital papillae on segments 11, 13–16, 23, 24, 27, 28, 36 *ab*.

Internal characters. Dissepiments 5/6–8/9 strongly thickened, 9/10 slightly thickened. Testes

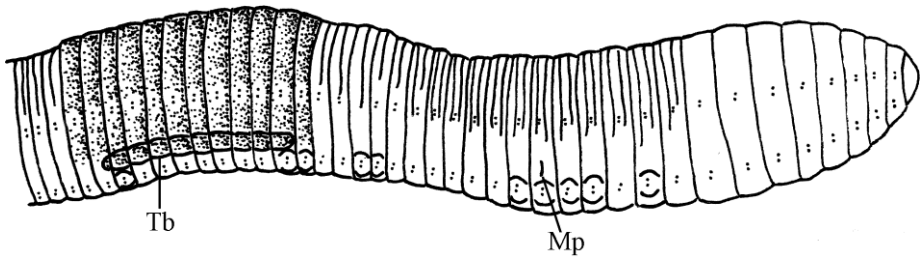

Figure 4. *A. sturanyi biharica* ssp. nov. Ventrolateral view of the clitellar region. Mp = male pore, Tb = tubercle.

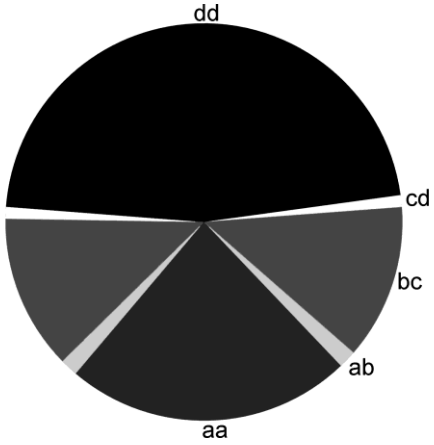

Figure 5. *A. sturanyi biharica* ssp. nov. Setal arrangement.

and funnels paired in segments 10–11, covered by perioesophageal testis sac. Seminal vesicles in 11, 12. Spermathecae two or three pairs in 9/10−10/11(11/12) with external openings near the setal line *cd*. Calciferous glands with well-developed diverticula in segment 10. Paired hearts appear in segments 6–11, with a pair of small extraoesophageal vessels in 12. Nephridial bladders J-shaped with proclinate ental part. Crop in segments 15–16, and gizzard in segments 17–18. Typhlosolis bifid, longitudinal musculature is of fasciculated type.

Etymology. The species name refers to the type locality.

Remarks. *A. s. biharica* ssp. nov. is similar to *A. s. sturanyi* and *A. s. dacidoides* in having 2/3 pairs of spermathecae but differs from both in the position of the clitellum and tubercles (Table 3) and also 11.5% and 10.6% K2P genetic distances respectively.

DISCUSSION

Integrated taxonomy proved to be highly efficient in recognizing and expanding the previously defined *Allolobophora sturanyi species* group (Csuzdi & Pop 2008). Both molecular taxonomic methods applied (Bayesian and ML analysis) highly supported the clade of the *A. sturanyi* subspecies (99% and 84% respectively) and also there is a moderate support for inclusion of the *A. gestroides* and *A. zicsica* species pair into the *sturanyi* species group. This inclusion is supported by morphology as well (Table 2).

Table 2. Distinguishing characters of the species in the *A. sturanyi* species group.

Species/subspecies	Clitellum	Tubercles	Receptacles	Segments
A. sturanyi sturanyi	27, **28**–*1/n* 39, **39**	**29**–38, ***1/n*39**	**3,** 9/10–11/12	170–210
A. sturanyi dacica	28, **29**–**37,** 38, 39	**30**–**37**, *1/n*38	**5,** (4) 9/10–12/13,13/14	165–182
A. sturanyi dacidoides	*1/n*27, **27**–**36**, 37	*1/n*28, **28**–**36**, 37	**3,4,** 9/10–11/12, 12/13	119–152
A. sturanyi biharica ssp. nov.	*1/n*27, **27**–**38**	**28**—**½37**, 37	**2,3,** 9/10–10/11, 11/12	205
A. prosellodacica	*1/n*24, **25**–½36, **36**	**30**–**½35**, 35	**3** 9/10–11/12	160–170
A. gestroides	29, **30**–**40**, 41	**35**–**40**, 41	**3,** 9/10–11/12	163–201
A. zicsica sp. nov.	**32**–**40**	**½35 –½40**	**3,** 9/10–11/12	125

It is interesting to note that the other Dacian species analysed (*Cernosvitovia opisthocystis*, *A. robusta* and *A. mehadiensis*) formed a well-supported (100% and 0.95) clade with the *sturanyi* species group (clade *a* in Fig. 1). This Balkanic-Central European clade of the *Allolobophora* species has already been recognized by Pop *et al.* (2005) and more recently in Domínguez *et al.* (2015). This clade (*a* here, and *c* in Domínguez *et al.* 2015) contains species relegated previously into different genera by Mršić (1991) i.e. *Cernosvitovia* (*opisthocystis, rebeli, dudichi*), *Karpatodinariona* (*dacica, dacidoides, sturanyi*), *Serbiona* (*mehadiensis, robusta*) and *Alpodinaridella* (*gestroides, gestroi*). According to the accumulating morphological and molecular results it seems that these species form a well separated Balkanic-Central European genus, different from *Allolobophora* proper. As the type species of the senior synonym of *Cernosvitovia* is also involved (*C. rebeli* (Rosa, 1897)) the valid name of this genus should be *Cernosvitovia*.

It is also remarkable that the *Helodrilus* and *Proctodrilus* species also formed a monophyletic clade (*b*) with quite high Bayesian and bootstrap support (89% and 81% respectively) and *Helodrilus* (clade *c*) and *Proctodrilus* (clade *d*) proved to be exclusively monophyletic.

The closer relationship of *Aporrectodea dubiosa* (Örley, 1881) and *Aporrectodea molleri* (Rosa, 1889) was yet observed (Pop *et al.* 2005) and here they grouped together with the other *Aporrectodea* species (clade *e*) unlike to the trees in Domínguez *at al.* (2015) where they formed a clade with *Allolobophora chlorotica* (Savigny, 1826) (type species of the genus). *Ap. dubiosa* and *Ap. molleri* seems to be close also morphologically, both species are relatively large-bodied (120–150 mm), greenish, possess fasciculated musculature and backward shifted clitellum terminating after segment 45.

Qiu & Bouché (1998) separated *Ap. molleri*, *Ap. dubiosa* and several other large-bodied greenish Franco-Iberian species into the newly erected *Heraclescolex*. If further molecular studies with more complete taxon sampling and wider gene selection prove their distinctness, the valid genus name would be *Archeodrilus* Szüts, 1913 with type species *Criodrilus dubiosus* Örley, 1881 by priority.

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