



## Two new species of *Bimastos* (Oligochaeta, Lumbricidae) from the Southern Appalachian Mountains, North America

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### Abstract

Two new species of *Bimastos* Moore are described based on morphological and molecular data. *Bimastos nanae* n. sp. resembles *B. lawrenceae* Fender, *B. zeteki* (Smith and Gittins) and *B. welchi* (Smith). *Bimastos nanae* n. sp. differs from these species in the position of the clitellum, size and number and position of thickened septa. *Bimastos magnum* n. sp. is similar to *B. schwerti* Csuzdi & Chang and *B. palustris* Moore in having a fully annular clitellum and male pores on huge porophores. *Bimastos magnum* n. sp. differs from both species by having a more posterior position of the clitellum (in xxiv–xxxiii, xxxiv) and larger body size. With the description of these new species, the number of *Bimastos* species is raised to 14.

**Key words:** Earthworm, North American native species, Fire regime, Male pores, *Bimastos*

### Resumen

Se describen dos especies nuevas de *Bimastos* Moore en base de datos morfológicos y moleculares. *Bimastos nanae* n. sp. se asemeja a *B. lawrenceae* Fender, *B. zeteki* (Smith & Gittins) y *B. welchi* (Smith). *Bimastos nanae* n. sp. se diferencia de estas especies en la posición de su clitelo, tamaño y localización de los tabiques engrosados. *Bimastos magnum* n. sp. se asemeja a *B. schwerti* Csuzdi & Chang y a *B. palustris* Moore al tener un clitelo completamente anular y sus poros masculinos en poróforos agrandados. *Bimastos magnum* n. sp. se diferencia de ambas especies al tener clitelo localizado posteriormente (en xxvi–xxxiii, xxxiv) y en tener un tamaño mayor. Con las descripciones de estas dos nuevas especies, el número de especies reconocidas de *Bimastos* incrementa a 14.

**Palabras Clave:** Lombrices de tierra, Especies nativas de Norte América, Régimen de fuego, Poros masculinos, *Bimastos*

### Introduction

*Bimastos* Moore, 1893 consists of twelve valid species, with most of its diversity concentrated in the midwestern and eastern United States. However, some species have become cosmopolitan throughout the world (Csuzdi *et al.* 2017). This has made classification and definition of *Bimastos* historically problematic, with the genus sometimes relegated to subgenus status within *Allolobophora* or *Helodrilus* (Michaelsen 1899, 1900; Smith 1917), or having its species placed variously in the genera *Eisenia*, *Dendrobaena*, *Allolobophoridella* and *Dendrodrilus* (Mršić 1990, 1991; Omodeo 1956; Pop 1941; Zicsi 1981). *Bimastos* differs from *Eisenia* and *Dendrobaena* in having U-shaped nephridial bladders, while *Allolobophoridella* and *Dendrodrilus* have been synonymized with *Bimastos* (Csuzdi *et al.* 2017).

*Bimastos* is characterized by having considerable reduction to loss of sexual organs like the spermathecae and the tubercula pubertatis. Reductions of these structures have evolved independently in the genera *Healyella* Omodeo and Rota, 1989, and *Spermophorodrilus* Bouché, 1975 (Csuzdi *et al.* 2017). However, *Bimastos* is closest to *Eisenoides* Gates, 1969, forming a single clade containing all North American endemic lumbricids (Domínguez *et al.* 2015; Csuzdi *et al.* 2017). Because of the reduction of sexual organs characteristic of the genus and ambiguous descriptions of some species, the number of accepted species of *Bimastos* has varied between authors over time. A systematic revision by Csuzdi *et al.* (2017) revealed that the genus contains 12 described species and provided redefinitions of most species based on type series materials. Recent collections in the Appalachian Mountains in North America revealed two undescribed species.

The Appalachian Mountain chain is considered a biodiversity hotspot (Luna-Vega & Contreras-Medina 2010; Milanovich *et al.* 2010; Tripp *et al.* 2019). This region currently has reported over 25 native species of earthworms (Reynolds 2017a; b), including at least seven known *Bimastos* species. This region has a long history of fire suppression, which allowed the accumulation of leaf litter that ignited in 2016 as a result of human agency and extremely dry condition (Carpenter *et al.* 2021). Therefore, understanding the diversity of this region is important to promote its conservation, especially given that environmental disturbance events are expected to increase in frequency in the near future (Jolly *et al.* 2015). During 2017 and 2018, efforts to understand the effects of these two large wildfires in northern Georgia, USA, on soil invertebrates revealed the presence of two undescribed *Bimastos* species. Both species are herein described using morphological and molecular data.

## Methods

The specimens were killed with 70–95% ethanol. Then we took body wall tissues samples (ca. 1 mm<sup>2</sup>) for future DNA extraction before the specimens were fixed in 10% formalin for at least 24hrs. The specimens were examined externally and internally by dorsal dissection, using the taxonomy presented by Csuzdi *et al.* (2017). Species length, width, and number of segments are presented in a range from all the type material studied. All specimens were deposited in the Georgia Museum of Natural History's Grace Thomas Invertebrate Collection (GTIC).

We extracted the DNA using the Qiagen™ DNeasy® Blood & Tissue DNA extraction kit and quantified DNA concentrations using NanoDrop (NanoDrop, Thermo Fisher Scientific Inc.). We amplified the ribosomal 16S gene using the primers 16S-tRNA-Leu-Ala-Ser-Leu-LumbF2 [5'-CGA CTG TTT AAC AAA AAC ATT GC-3', Pérez-Losada *et al.* (2009)], and Ho\_16SRa [5'-GCA CTA TTC TGC CAY CTT GT-3', Novo *et al.* (2010)], and the mitochondrial Cytochrome c Oxidase subunit I (COI) barcode gene using the primers LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'), following Folmer *et al.* (1994). We performed PCR reactions at 25 µL final volume of 12.5 µL of GoTaq® Colorless Master Mix (Promega; composed by 2X Colorless GoTaq® Reaction Buffer of pH 8.5, 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl<sub>2</sub>), 1.0 µL of BSA (1x), and 1.0 µL of each of forward and reverse primers (10 µM) and of template DNA. An additional 1.0–2.5 µL of MgCl<sub>2</sub> (25mM) was added to the 16S reactions. The PCR cycling conditions differed for both markers, and we optimized them with the following steps: for COI; PCR cycling conditions were one cycle at 95°C for 2 min, 40 cycles at 95°C for 30 seconds, 50°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 7 min., for 16S: PCR cycling conditions were one cycle at 95°C for 2 min, 35–40 cycles at 95°C for 90 sec, 60°C for 1 min, and 72°C for 90 sec, with a final extension at 72°C for 7 min. We ran PCR products through a 1.3% agarose gel electrophoresis to confirm successful amplification before cleaning-up the PCR products.

The PCR products were cleaned with 1.5 µL of Exo-Sap-IT (Affymetrix) (1:5 dilution with PCR-grade water) and incubated at 37°C for 30 min followed by 80°C for 15 min. Then, the Exo-Sap IT cleaned samples were prepared for cycle sequencing in both primer directions, by adding 2.0 µL of the DNA to a mix of 0.5 µL BigDye 3.1 (Applied Biosystems), 0.5 µL of 5x Sequencing Buffer (Applied Biosystems), 0.33 µL of 10 µM Primer (of one direction only), and 7.5 µL ddH<sub>2</sub>O, for a total volume of 10.83 µL. The samples were then run through the cycle sequence with an initial denaturation at 95°C for 2 min, followed by 25 cycles consisting of denaturation at 95°C for 15 sec, annealing at 50°C for 15 sec and extension at 60°C for 4 min. The samples were then prepared on a plate and prepared to be sent for sequencing to the Center of Biotechnology of the University of Wisconsin-Madison, using ABI 3730xl Capillarity Sequencer (<https://biotech.wisc.edu/>).

Sequences were aligned using M-Coffee (Wallace *et al.* 2006), to examine alternative alignment algorithms and finding the best approach. M-Coffee combines eight commonly used alignment algorithms to identify the preferred alignment approach and provides information on the final alignment quality by calculating a score for each individual sequence with respect of the other sequences and the overall alignment quality (Wallace *et al.* 2006). Both alignments had an overall score of 99 and 96 for COI and 16S, respectively, with individual sequences scoring  $\geq 96$  on COI, and  $\geq 93$  on 16S. Sequences were trimmed using trimAl (Capella-Gutiérrez *et al.* 2009) using 5 bp windows, maintaining a minimum of 40% similarity at each base position, and allowing gaps. Trimming resulted in a sequence length of 648 bp for COI and 489 bp for 16S. Genetic divergence was calculated using Kimura 2—Parameter model (Kimura 1980) with complete deletion on MEGA X (Kumar *et al.* 2018) using the *Bimastos* sequences available on GenBank, generated by Csuzdi *et al.* (2017) and Domínguez *et al.* (2015). Additional specimens deposited in the GTIC of *B. zeteki* (GTIC-10325 and 10326), *B. longicinctus* (GTIC-10684) and *B. tumidus* (GTIC-11346 to 11348) were studied. Sequences for the *B. zeteki* and *B. longicinctus* specimens were generated by Ikeda *et al.* (2020) and downloaded from GenBank.

For the phylogenetic analysis, we included sequences of *Eisenoides caroliniensis*, and *E. lonnbergi* as putative sister taxa of *Bimastos*. For a more distant outgroups, we used *Haelyella jordanis*, *H. syriaca*, *Fitzingeria platyura platyura*, *Spermaphorodrilus antiguus*, and *Dendrobaena alpina* with sequences generated by Csuzdi *et al.* (2017) and Domínguez *et al.* (2015). The newly and previously generated sequence accession numbers are available in Appendix 1. We concatenated the COI and 16S sequences with MEGA X (Kumar *et al.* 2018) for a total length of 1137 bp. We used ModelFinder (Kalyaanamoorthy *et al.* 2017) in IQ-TREE ver. 1.6.12 (Nguyen *et al.* 2015) to determine the most appropriate substitution model based on the corrected Akaike Information Criterion (cAIC). The best substitution model was GTR+F+I+G4 for COI and TIM2+F+I+G4 for 16S, and we used RAXML-NG ver. 1.0.2 (Kozlov *et al.* 2019) to generate the phylogenetic tree implementing this preferred substitution model. Branch support was provided by the Transfer Bootstrap Expectation (TBE), as it performs better on deep branches, and rarely supports weak branches compared to the traditional Felsenstein Bootstrap Proportions (FBP) (Lemoine *et al.* 2018). A final tree image was generated using ggtree ver. 3.0.2 (Yu *et al.* 2017) in R ver. 4.1.0 (R Core Team 2021).

## Results

Both new species are supported by a divergence (presented as the proportions of base pair differences) of 0.127–0.185 at the COI locus (Table 1), and of 0.054–0.138 in 16S sequences (Table 2). Within species distances for *Bimastos magnum* and *B. nanae* were 0.060 (SE+/- 0.011) and 0.019 (SE+/- 0.006) for COI, and 0.073 (SE+/- 0.011) and 0.038 (SE+/- 0.010) for 16S, respectively.

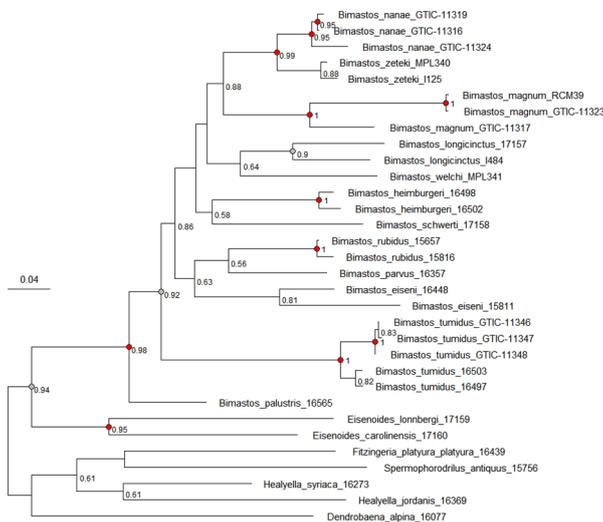
The phylogeny confirmed the monophyly of both *B. nanae* and *B. magnum*. The analysis suggests that *B. nanae* is sister taxon for *B. zeteki*, while *B. magnum* was paired with *B. nanae* + *B. zeteki* clade (Figure 1).

**TABLE 1.** COI Kimura two-parameter (2-p) divergence distances (lower-left) and standard errors of each comparison (upper-right) between known species of *Bimastos* (for which sequence data are available). Divergence distances are expressed as the proportion of base pair differences.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
1. <i>B. eiseni</i>		0.022	0.019	0.020	0.019	0.023	0.020	0.021	0.022	0.021
2. <i>B. heimbürgeri</i>	0.175		0.020	0.020	0.019	0.023	0.021	0.023	0.019	0.021
3. <i>B. longicinctus</i>	0.166	0.155		0.022	0.019	0.019	0.018	0.022	0.020	0.018
4. <i>B. palustris</i>	0.152	0.137	0.171		0.021	0.021	0.022	0.021	0.021	0.020
5. <i>B. parvus</i>	0.144	0.127	0.144	0.127		0.023	0.019	0.021	0.019	0.019
6. <i>B. schwerti</i>	0.169	0.175	0.154	0.164	0.147		0.024	0.023	0.022	0.021
7. <i>B. tumidus</i>	0.170	0.156	0.142	0.136	0.154	0.196		0.020	0.021	0.021
8. <i>B. rubidus</i>	0.177	0.178	0.183	0.139	0.134	0.176	0.147		0.022	0.021
9. <i>B. magnum</i>	0.185	0.144	0.168	0.139	0.153	0.168	0.159	0.177		0.019
10. <i>B. nanae</i>	0.157	0.145	0.147	0.126	0.128	0.140	0.166	0.151	0.140	

**TABLE 2.** 16S Kimura two-parameter (2-p) divergence distances (lower-left) and standard errors of each comparison (upper-right) between known species of *Bimastos* (for which sequence data are available). Divergence distances are expressed as the proportion of base pair differences.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
1. <i>B. eiseni</i>		0.013	0.013	0.015	0.011	0.014	0.016	0.012	0.016	0.014	0.017	0.016
2. <i>B. heimbürgeri</i>	0.079		0.013	0.013	0.013	0.015	0.015	0.013	0.014	0.010	0.013	0.013
3. <i>B. longicinctus</i>	0.083	0.091		0.015	0.012	0.015	0.015	0.015	0.012	0.013	0.014	0.015
4. <i>B. palustris</i>	0.106	0.108	0.123		0.015	0.017	0.016	0.011	0.016	0.013	0.015	0.017
5. <i>B. parvus</i>	0.085	0.099	0.101	0.111		0.014	0.015	0.011	0.015	0.014	0.017	0.015
6. <i>B. schwerti</i>	0.092	0.081	0.109	0.106	0.110		0.018	0.015	0.017	0.015	0.015	0.015
7. <i>B. tumidus</i>	0.123	0.116	0.121	0.121	0.112	0.125		0.016	0.018	0.015	0.016	0.016
8. <i>B. rubidus</i>	0.082	0.092	0.104	0.092	0.060	0.102	0.130		0.015	0.013	0.014	0.015
9. <i>B. welchi</i>	0.108	0.098	0.088	0.111	0.103	0.122	0.132	0.093		0.014	0.016	0.017
10. <i>B. zeteki</i>	0.095	0.073	0.092	0.103	0.098	0.091	0.126	0.091	0.098		0.013	0.011
11. <i>B. magnum</i>	0.130	0.109	0.112	0.135	0.138	0.122	0.151	0.138	0.134	0.110		0.015
12. <i>B. nanae</i>	0.102	0.085	0.104	0.125	0.103	0.099	0.119	0.095	0.106	0.054	0.123	



**FIGURE 1.** Maximum Likelihood phylogenetic analysis of the concatenated 16S and COI genes. Branch support are Transfer Bootstrap Expectation (TBE), nodes with a light grey circle have TBE  $\geq 0.90$ , with red circles (dark grey in printed version),  $\geq 0.95$ . Nodes without numbers or circles have TBE  $< 0.50$ . Branch length represents number of substitutions per site, given the available data (see Appendix 1).

## Taxonomy

### *Bimastos* Moore, 1893

For diagnosis and full list of synonyms for the genus, see Csuzdi *et al.* (2017).

#### *Bimastos nanae* Carrera-Martínez n. sp.

Figure 2 A, C

urn:lsid:zoobank.org:act:907B4103-225D-41F5-BC5B-37C3EB3A421B

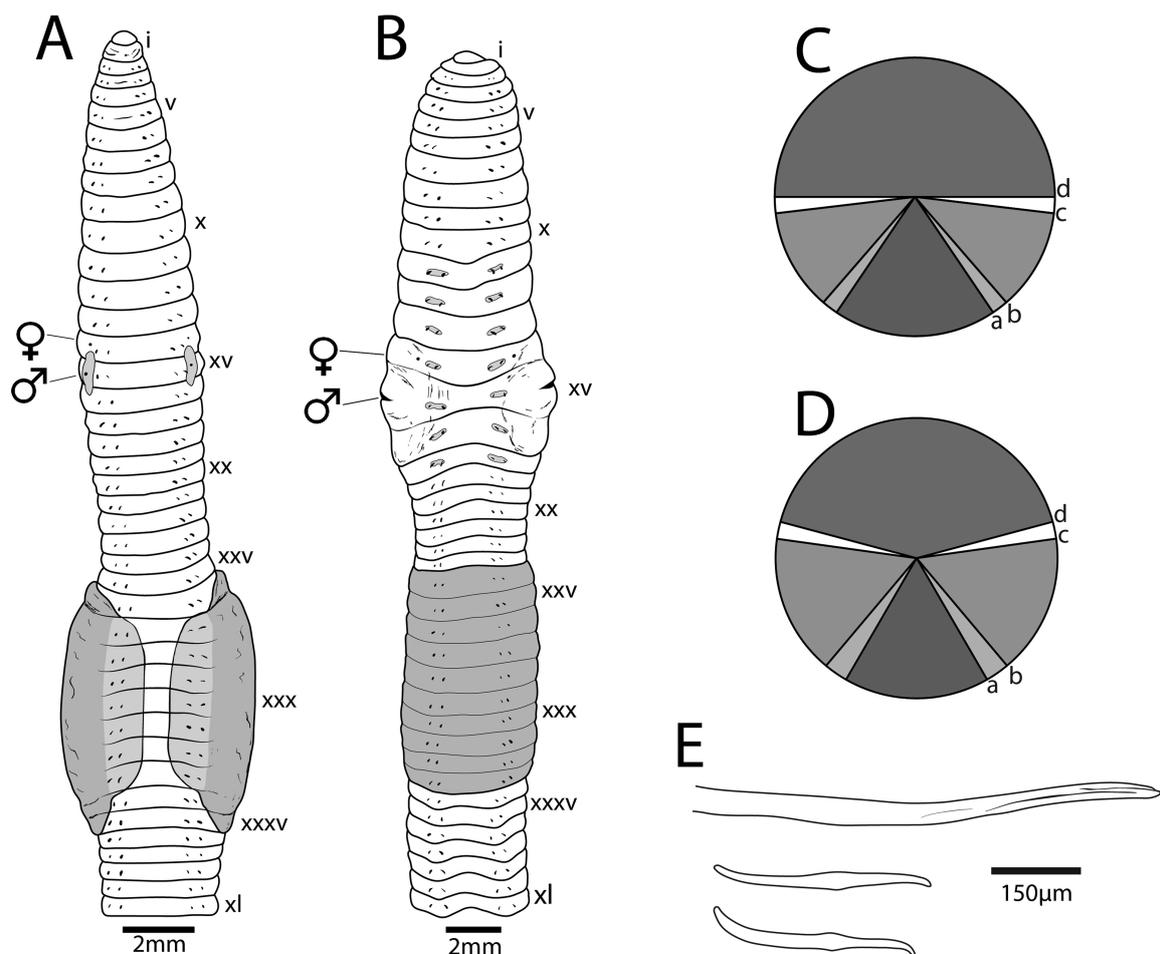
Holotype. Adult (GTIC-11519), collected from topsoil, under mosses and leaf litter in saturated soil near Dismal Creek at 730–800 m elevation, Chattahoochee National Forest, Towns Co., GA, USA (34.893879N, 83.660047W), Cols. SW James, MA Callaham Jr, MK Taylor, BA Snyder, R Carrera-Martínez, 26.SEP.2017.

Paratypes. One adult collected with Holotype (GTIC-11516). One early adult (GTIC-11540) collected by grunting near Bombing Rd, south of Isaacs Creek at 140 m elevation, Sumter National Forest, Union Co., SC, USA (34.647609N, 81.732213W), Cols. R Carrera-Martínez, MK Taylor, MA Callaham Jr, 07.MAR.2017. One adult (GTIC-11522), ca. 600 m west of Tate City Rd. and 320 m east of Sassafras Branch, at 1,012 m altitude, Nantahala National Forest, Clay Co., NC, USA, Cols. MK Taylor, GL Chapman, DO Carpenter, 21.NOV.2018. One adult (GTIC-11524) Quiet Walkway, N of Carlos Campbell Overlook, GSMNP, Sevier Co. TN., USA, Cols. MA Callaham, MK Taylor, 01.NOV.2017.

Etymology: This species is named in loving memory of Nan Christine Hediger, mother of Dana O. Carpenter, who collected part of the type series.

### External characteristics (Figure 2A):

Length 58–80 mm. Diameter at x 3.4–5.0 mm, at the clitellum 5.0–6.0 mm. Number of segments 130–149, simple annulation. Live specimen brown; preserved, body light violet-reddish dorsally, clitellum pale to pinkish-white. Prostomium epilobic, narrow and open, extends through about 2/3 of the peristomium. First dorsal pore in 4/5 or 5/6. Spermathecal pores absent. Female pores in xiv, lateral and next to *b*, small but visible. Male pores equatorial and conspicuous with associated swelling between *bc*, closer to *b*. Clitellum in xxvi–xxxv, saddle-shaped, ventral limit in *b* in xxvi–xxvii, *a* in xviii–xxxv. Tubercula pubertatis absent. No genital markings were observed. Nephridiopores, when visible, forming a regular row dorsal to *d*. Setae, lumbricine, 8 per segment, closely paired. In x *aa:ab:bc:cd:dd* = 5.21:1.17:3.75:1.00:13.33, in xl = 10.00:1.00:6.25:1.00:26.50; *cd* at the mid-lateral line throughout (Figure 2C). All setae unmodified.



**FIGURE 2.** External morphology of (A) *Bimastos nanae* n. sp. and (B) *Bimastos magnum* n. sp. based on their holotypes; setae distribution in xl of (C) *B. nanae* n. sp. and (D) *B. magnum* n. sp.; and (E, top) distal end of the copulatory setae of segment xv of *B. magnum* n. sp. holotype and somatic setae *ab* of xviii (E, center and bottom), scale on E is the same for all three setae. In A and B, ♀: female pore and ♂: male pore.

## Internal anatomy:

Septa 5/6–10/11 pushed backwards, 6/7–8/9 thickened, 5/6 and 9/10 slightly thickened, 15/16–17/18 very thin and delicate. Pharynx ends in iv, associated with many muscular fibers attached to body wall. Calciferous sacks medium to large in x, calciferous glands in xi–xii not interrupted by 11/12 septum. Crop in xiv–xv, subdivided into two sections of similar size by septum 14/15. Gizzard, large and muscular in xvii–xviii, valve in 18/19. Intestinal origin abrupt in xix. Typhlosole arising in xxi, bifid or Y-shaped, increasing in size progressively, occupying the entire lumen of the intestine in the posterior half of the body, before ending abruptly in the region of xciii. Hearts in vii–xi, largest in x. Dorsal and ventral trunks simple, dorsal first visible at vii. Anterior to vii, only small lateral commissures can be observed. Holonephridia paired, one per segment, with tubular bladder U-shaped with proclinate ental limb, extending to *d*, parallel to the much thinner vesicles, with constant shape and size throughout the body. Spermathecae absent. Ovaries, bundle-like in xiii. Testes free in x and xi. Seminal vesicles large and free, covering the intestinal tract completely in xi and xii only. Prostates absent. No iridescence observed in reproductive organs.

Remarks: *Bimastos nanae* n. sp. resembles *B. lawrenceae* Fender 1994, *B. zeteki* (Smith & Gittins 1915) and *B. welchi* (Smith 1917), since all have the clitellum located in the same general area. However, *B. nanae* has its clitellum located in xxvi–xxxv, whereas *B. lawrenceae* has it in xxv–xxxiv, *B. zeteki* in xxvii–xxxvii, xxxviii and *B. welchi* in xxv–xxxv. Additionally, *B. nanae* differs from *B. zeteki* and *B. lawrenceae* in having less pigmentation and from *B. zeteki* and *B. welchi* on having a smaller size. The size and number of segments of *B. lawrenceae* is not mentioned in its original description, but the shorter extension of the calciferous glands in *B. nanae*, different typhlosolar origin (xxi in *B. nanae* vs. xxii–xxiii in *B. lawrenceae*), different location of thickened septa (7/8–8/9 in *B. nanae*, vs. 12/13–14/15 only slightly in *B. lawrenceae*), and different setal ratios can be used to differentiate both species. Table 3 summarizes differences between these species.

**TABLE 3.** Differences between *Bimastos nanae* n. sp. and morphologically similar species. Morphological data for all previously described species obtained from Csuzdi *et al.* (2017) and their original descriptions (McKey-Fender *et al.* 1994; Smith 1917; Smith & Gittins 1915). Question marks represent unknown characters.

Characters	<i>B. nanae</i> n. sp.	<i>B. lawrenceae</i> Fender, 1994	<i>B. welchi</i> Smith, 1917	<i>B. zeteki</i> Smith & Gittins, 1915
Length (mm)	58–80	?	135	100–140
Diameter (mm)	4.5–6.0	?	4.5	5.0–6.5
No. of segments	130–150	?	116	134–142
Pigmentation	Light violet	Red	Pale	Violet
Clitellum	xxvi–xxxv	xxv–xxxiv	xxv–xxxv	xxvii–xxxvii, xxxviii
Setal ratio	$aa > bc$ , $ab \approx cd$	$aa \approx bc$ , $ab \approx cd$	$aa > bc$ , $ab > cd$	$aa > bc$ , $ab = cd$
Thickened septa	6/7–8/9 strongly, 5/6, 9/10 slightly	12/13–14/15 moderately	8/9–11/12 strongly, 6/7–7/8, 12/13 slightly	7/8–12/13 slightly, 13/14–14/15 moderately
Calciferous glands	xi–xii, medium to large diverticula in x	xi–xiii, diverticula in x	xi–xii, small diverticula in x	xi–xii, small diverticula in x
Typhlosole	Bifid or Y-shaped	Bifid or Y-shaped	?	Lamelliform

### *Bimastos magnum* Carrera-Martínez n. sp.

Figure 2 B, D, E

urn:lsid:zoobank.org:act:E3A5D18A-40D1-4C25-80C8-E978ADAF9CD2

Holotype. One adult (GTIC-11517), collected from topsoil, under mosses and leaf litter in saturated soil near Dismal Creek at 730–800 m elevation, Chattahoochee National Forest, Towns Co., GA, USA (34.893879N, 83.660047W), Cols. SW James, MA Callaham, MK Taylor, BA Snyder, R Carrera-Martínez, 26.SEP.2017.

Paratype. Two adults (GTIC-11518, GTIC-11520), collected with Holotype. One adult (GTIC-11523), Chattahoochee NF, Murray Co., GA, USA (34.8664N, 84.6438W), Cols. MA Callaham, MK Taylor, 05.OCT.2017.

Etymology: From *magnus*, Latin for large, or huge, in reference to its generally large size relative to other species in the genus, and specifically to its male pores and porophores.

**TABLE 4.** Differences between *Bimastos magnum* n. sp. and other *Bimastos* species with annular clitellum. Morphological data for *B. palustris* obtained from Moore (1895) and Csuzdi *et al.* (2017) and for *B. schwerti*, from Csuzdi *et al.* (2017).

Characters	<i>B. magnum</i> n. sp.	<i>B. palustris</i> Moore, 1895	<i>B. schwerti</i> Csuzdi and Chang, 2017
Length (mm)	65–120	18–30	25–62
Diameter (mm)	4.2–4.8	1.5–2.5	2.0–5.0
No. of segments	118–152	80–100	88–120
Pigmentation (preserved)	Reddish dorsally	Pale with reddish dorsum	Brown (red-violet alive)
Clitellum	xxiv-xxxiii, xxxiv	xxiii-xxviii	xxi, xxii, xxiii-xxx
Setal ratio	$aa \approx bc, ab > cd$	$aa > bc, ab > cd$	$aa \approx bc, ab > cd$
Copulatory setae	xi–xvii	Absent	Absent
Thickened septa	5/6–12/13 thickened, 4/5, 13/14–14/15 slightly	7/8–8/9 slightly	6/7–8/9, 12/13–14/15 moderately; 9/10–10/11 slightly
Calciferous glands	xi-xii, (1/n) xiii, large diverticula in x	x-xii, moderate diverticula in x	x-xiii, small diverticula in x
Typhlosole	Bifid or Y-shaped	Lamellar	Bifid or Y-shaped

### External characteristics (Figure 2B):

Length 66–120 mm. Diameter at x 4.5–4.6 mm, at the clitellum 4.2–4.8 mm. Number of segments 118–152, vi–xvii with dorsal secondary annulation between *dd*. Live specimen red to brown-red; preserved, body and clitellum almost completely white, reddish on the anterior dorsum. Prostomium epilobic, narrow and open, extends through about 2/3 of the peristomium. First dorsal pore small in 4/5, after 5/6, dorsal pores larger. Spermathecal pores inconspicuous. Female pores in xiv, next to *b*, slightly presetal, conspicuous. Male pores equatorial, conspicuous with associated porophores extending into part of xiv and xvi, midway between *bc*. Clitellum in xxiv-xxxiii, xxxiv, annular, uniformly developed ventrally. Tubercula pubertatis absent. Genital markings surrounding *ab* on xi-xvii, xviii. Nephridiopores, when visible, at or dorsal to *d*. Setae, lumbricine, 8 per segment, closely paired: in x  $aa:ab:bc:cd:dd = 8.56:1.44:9.44:1.00:23.89$ , in xl =  $8.50:1.40:8.22:1.00:21.22$  (Figure 2D). Setae all small, difficult to observe externally, smooth, ca. 300  $\mu$ m long and 35  $\mu$ m wide; *ab* modified as copulatory setae in xi-xvii; in xv, ca. 1.3 mm long, 50–51  $\mu$ m midpoint diameter, smooth throughout, blunt tip (Figure 2E).

### Internal anatomy:

Septa 4/5, 13/14–14/15 slightly thickened, 5/6–12/13 thickened. Well-developed pharynx ends in iv. Calciferous diverticula huge in x, calciferous glands in xi-xii, partially extending into xiii. Crop in xiv-xv, subdivided into two sections of similar size by septum 14/15. Gizzard large and muscular in xvi-xvii, valve in 17/18. Intestinal origin abrupt in xviii. Typhlosole arising in xviii, bifid or Y-shaped, increasing in size progressively, occupying about 1/3 of the diameter of the lumen of the intestine at xxv. Hearts in vii-xi, largest in x. Dorsal and ventral trunks simple. Anterior to vii, small lateral commissures present. Holonephridia paired, one per segment, with tubular bladder U-shaped, extending dorsal to *d*, parallel to the much thinner vesicles, posterior to xv, bladder progressively gains a long J-shape. Paired spermathecae present in xiii-xvii, ventral. Ovaries, fan-like in xiii. Testes free in x and xi. Seminal vesicles big and free, covering the intestinal tract completely in x, xi and xii, iridescent. Prostates absent.

Remarks: *Bimastos magnum* n. sp. is the only known species in the genus to possess copulatory setae and spermathecae; it is the third possessing a completely annular clitellum and male pores on large porophores extending into adjacent segments, with the other two being *B. palustris* Moore, 1895 and *B. schwerti* Csuzdi and Chang, 2017. *Bimastos magnum* differs from these species in the position of its clitellum (xxiv-xxxiii, xxxiv) and size (65–120

mm) as both, *B. palustris* and *B. schwerti*, have a more anteriorly placed clitellum (xxiii-xxviii and xxiii-xxx, respectively) and are smaller (18–30 and 25–62 mm, respectively). Table 4 summarizes differences between these species. *Bimastos magnum* is among the largest known species of the genus, comparable to, or only slightly smaller than *B. zeteki* and *B. welchi*. *Bimastos magnum* is the second *Bimastos* species to have spermathecae, with *B. rubidus* occasionally presenting spermathecae in ix and x. It is unknown how prevalent, functional, or variable are the presence of spermathecae in *B. magnum*, some of which were iridescent.

## Discussion

Following the key to *Bimastos* species from Csuzdi *et al.* (2017), *B. nanae* n. sp. will key to couplet 5, where *B. welchi* and *B. lawrenceae* are the only options. *Bimastos nanae* n. sp. differs from these species by having the clitellum in xxvi-xxxv, a ratio between *ab:cd*, and in the location of the thickened septa (Table 3). *Bimastos magnum* n. sp. will key to couplet 2 in Csuzdi *et al.* (2017), where *B. palustris* and *B. schwerti* are the only options. *Bimastos magnum* n. sp. differs from both species by its larger size (65–120 mm) and in having the clitellum in xxiv-xxxiii, xxxiv (Table 4). *Bimastos magnum* is unique in the genus by having copulatory setae and spermatheca. *Bimastos rubidus* occasionally has spermathecae, but these are usually small and empty, however no other known *Bimastos* species has copulatory setae. Nonetheless, the lack of tubercula pubertatis, having calciferous diverticula in x, and pigmentation and u-shaped nephridial bladder supports its placement within *Bimastos* (Csuzdi *et al.* 2017; Gates 1969), as supported by our phylogenetic analysis (Figure 1).

According to our phylogenetic inference (Figure 1), *B. nanae* is closest to *B. zeteki*, while *B. magnum* is the sister taxon to the clade formed by *B. nanae* and *B. zeteki* (although with relatively weak support). This suggests that the development of large porophores and presence of spermathecae has been either gained or lost multiple times within *Bimastos*. Nonetheless, we also note the relatively short branches (as seen in Csuzdi *et al.* 2017) and weak support (TBE < 0.90) for the deeper clades of the genus. This might indicate a rapid species radiation event, however further studies are needed to confirm this hypothesis and confirm the evolutionary history of the group.

With the description of *B. nanae* and *B. magnum*, the number of valid species of *Bimastos* is raised to 14 species (Csuzdi *et al.* 2017). Most of these species are found in eastern North America, including the two described here. *Bimastos welchi* Smith, 1917, is found in the Great Plains region of North America, whereas *B. lawrenceae* Fender, 1994, is the only species endemic to the Pacific Coast, where it is so far limited to Vancouver Island (Csuzdi *et al.* 2017; McKey-Fender *et al.* 1994; Smith 1917). Together, *Bimastos* and *Eisenoides* form a monophyletic group endemic to North America, and are sister taxa to the mostly temperate Eurasian *Eisenia* (Csuzdi *et al.* 2017). This divergence has been dated back to the Late Cretaceous (Domínguez *et al.* 2015) but there is still uncertainty about how the *Bimastos-Eisenoides* clade arrived in North America (Csuzdi *et al.* 2017). It is notable that the earthworm diversity of the Pacific Northwestern coast of North America has been sorely neglected for the last 30+ years, but there are reports of extensive, undescribed diversity present in that region (Fender 1995; Fender & McKey-Fender 1990). Additionally, many undescribed species of earthworms are still being collected (Csuzdi *et al.* 2017; Damoff & Reynolds 2017). Therefore, we expect that further collecting across North America will yield additional undescribed species that will be referable to the *Bimastos-Eisenoides* clade.

Both *B. nanae* and *B. magnum* (and most of the *Bimastos* species) occur in the Appalachian Mountains and Piedmont region. Several lines of evidence suggest that the ecosystems of the Southern Appalachians developed under the influence of frequent fires, and that most areas burned every 2–20 years. However, the recent (post-European settlement) history of the region has been one of nearly complete fire suppression (Flatley *et al.* 2013; Lafon *et al.* 2017). It therefore seems reasonable to suppose that any native earthworm fauna would be adapted to frequent fires, and indeed both specimens described here seem to have epi-endogeic habits which essentially protects them from receiving direct effects of fire (Certini *et al.* 2021; Ikeda *et al.* 2015). Interestingly, several species of *Bimastos* have the habit of living inside the bark of decomposing trees (thus, the common name of American log worm or bark worm [James 1995; Reynolds 1977]), and this too would likely be a low-risk place to weather low-intensity surface fires that are characteristic of frequently burned ecosystems. This open niche could have perhaps resulted in a species radiation event discussed earlier (Martin & Richards 2019). In any case, the effect of reintroducing fire into these ecosystems after decades of fire suppression on the earthworm fauna, native or introduced needs to be addressed (but see Ikeda *et al.* 2015).

**Appendix 1.** Accession number for all COI and 16S DNA sequences. Specimens with asterisks are the holotype of *B. magnum* and *B. nanae*. Numbers after specimens from Csuzdi *et al.* (2017) refers to their Hungarian Natural History Museum (HNHM) catalog numbers.

Species and specimen ID	COI	16S	Reference
<i>Bimastos eiseni</i> HNHM-15811	KX651115	-	Csuzdi <i>et al.</i> (2017)
<i>Bimastos eiseni</i> HNHM-16448	KX651116	KX651221	Csuzdi <i>et al.</i> (2017)
<i>Bimastos heimbürgeri</i> HNHM-16498	KX651119	KX651224	Csuzdi <i>et al.</i> (2017)
<i>Bimastos heimbürgeri</i> HNHM-16502	KX651120	KX651225	Csuzdi <i>et al.</i> (2017)
<i>Bimastos longicinctus</i> HNHM-017157	KX651137	KX651240	Csuzdi <i>et al.</i> (2017)
<i>Bimastos longicinctus</i> 1484	LC475696	LC476001	Ikeda <i>et al.</i> (2020)
<i>Bimastos magnum</i> GTIC-11517*	OK104083	OK143525	This study
<i>Bimastos magnum</i> GTIC-11523	OK104085	OK143527	This study
<i>Bimastos magnum</i> RCM39	OK104084	OK143526	This study
<i>Bimastos nanae</i> GTIC-11516	OK104086	OK143523	This study
<i>Bimastos nanae</i> GTIC-11519*	OK104088	-	This study
<i>Bimastos nanae</i> GTIC-11524	OK104087	OK143524	This study
<i>Bimastos palustris</i> HNHM-16565	KX651121	KX651226	Csuzdi <i>et al.</i> (2017)
<i>Bimastos parvus</i> HNHM-16357	KX651122	KX651227	Csuzdi <i>et al.</i> (2017)
<i>Bimastos rubidus</i> HNHM-15657	KX651130	KX651234	Csuzdi <i>et al.</i> (2017)
<i>Bimastos rubidus</i> HNHM-15816	KX651131	KX651235	Csuzdi <i>et al.</i> (2017)
<i>Bimastos schwerti</i> HNHM-17158	KX651138	KX651241	Csuzdi <i>et al.</i> (2017)
<i>Bimastos tumidus</i> HNHM-16497	KX651123	-	Csuzdi <i>et al.</i> (2017)
<i>Bimastos tumidus</i> HNHM-16503	KX651124	KX651228	Csuzdi <i>et al.</i> (2017)
<i>Bimastos tumidus</i> GTIC-11546	OK104080	-	This study
<i>Bimastos tumidus</i> GTIC-11547	OK104081	-	This study
<i>Bimastos tumidus</i> GTIC-11548	OK104082	-	This study
<i>Bimastos welchi</i> MPL241	-	KJ912520	Domínguez <i>et al.</i> (2013)
<i>Bimastos zeteki</i> 1125	-	LC475991	Ikeda <i>et al.</i> (2020)
<i>Bimastos zeteki</i> MPL340	-	KJ912521	Domínguez <i>et al.</i> (2013)
<i>Eisenoides carolinensis</i> HNHM-17160	KX651140	KX651243	Csuzdi <i>et al.</i> (2017)
<i>Eisenoides lonnbergi</i> HNHM-17159	KX651139	KX651242	Csuzdi <i>et al.</i> (2017)
<i>Healyella jordanis</i> HNHM-16369	KX651133	KX651237	Csuzdi <i>et al.</i> (2017)
<i>Healyella syriaca</i> HNHM-16273	KX651134	-	Csuzdi <i>et al.</i> (2017)
<i>Fitzingeria platyura platyura</i> HNHM-16439	KX651141	KX651245	Csuzdi <i>et al.</i> (2017)
<i>Spermophorodrilus antiquus</i> HNHM-15756	KX651136	-	Csuzdi <i>et al.</i> (2017)
<i>Dendrobaena alpina</i> HNHM-16077	KX651125	KX651229	Csuzdi <i>et al.</i> (2017)

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