



Molecular Survey of Earthworms (Annelida: Clitellata) in Southwestern Ohio, with Identification of a New Non-Native Species (Family Moniligastridae)

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Abstract: Earthworms were collected from beneath cover objects in wooded natural areas in southwestern Ohio, USA, from 2017 to 2019. We identified a sample of these earthworms using mitochondrial cytochrome oxidase subunit 1 (COI). Most were non-native species, especially lumbricids and to a lesser extent megascolecids. *Lumbricus castaneus*, *Amyntas tokioensis*, and potentially a species of *Sparganophilus* represent new records for Ohio. Additionally, we report a non-native moniligastrid (genus *Drawida*) previously unknown in continental North America. Earthworms native to North America (*Diplocardia* and *Sparganophilus*) represented the smallest portion of our sample, with no native lumbricid earthworm species detected.

Keywords: Annelida, earthworm, non-native species, *Drawida*, CO1 barcoding

INTRODUCTION

Earthworm diversity in Ohio and in the Cincinnati region is understudied, with the most systematic attempt to document Ohio's species by Olson (1928, 1933). Additional recent records are found in Porco et al. (2013) and Reynolds (2018). Ohio's earthworm diversity includes native and non-native species and is influenced by glaciation, anthropogenic disturbance, and human-mediated introductions (Hendrix et al. 2008). The Cincinnati region is an ideal location to study the impact of these factors because, in addition to being greatly impacted by human activity, it lies at the intersection of two level III North American ecoregions: the glaciated Eastern Corn Belt Plains and the mostly unglaciated Interior Plateau (Reynolds 2015).

Currently, eleven native species are known for these two ecoregions in Ohio, with eight in greater Cincinnati (Hamilton and adjacent counties in Ohio, Kentucky, and Indiana; Reynolds 1994, 2008, 2015). These species represent four basic ecotypes: epigeic earthworms in leaf litter at the soil surface, endogeic earthworms in upper soil layers, limicolous earthworms in water-saturated soils, and corticole earthworms in decaying wood (Reynolds 2018). In Ohio, most native species have epigeic, endogeic, or corticole lifestyles, with only the sparganophilids being limicolous.

Many non-native earthworms add to this native diversity, with a variety of Palearctic species (mostly from Lumbricidae and Megascolecidae) established in our region (Hendrix et al. 2008; Chang et al. 2021). Accidental and purposeful introductions continue today through such activities as bait

farming, vermicomposting, and plant importation. Many non-native earthworm species negatively impact North American ecosystems through changes in nutrient cycling, decreased soil fertility, increased erosion, and changes in competitive interactions (Hendrix et al. 2008). Introductions have added 12 non-native species to the Eastern Corn Belt Plains and Interior Plateau in Ohio, with ten known from counties in greater Cincinnati (Reynolds 1994, 2008, 2015).

We add to this existing work, focusing on the Cincinnati region. By virtue of its location at the northern edge of the Interior Plateau, Cincinnati would be expected to have a high diversity of native earthworms compared to more glaciated areas of Ohio. Additionally, the warm climate and high level of development and disturbance predict increased establishment of non-native species (Hendrix et al. 2008). Earthworm identification is challenging because taxonomically useful morphological characteristics are limited, especially for immature specimens. Thus, we employed molecular sequence analysis, which has been used in recent work to allow more precise identification of taxonomically problematic species, morphologically cryptic species, and ecologically distinct lineages (Martinsson et al. 2015).

METHODS

We collected earthworms from beneath cover objects (e.g., logs and rocks) from March through December of 2017–2019.

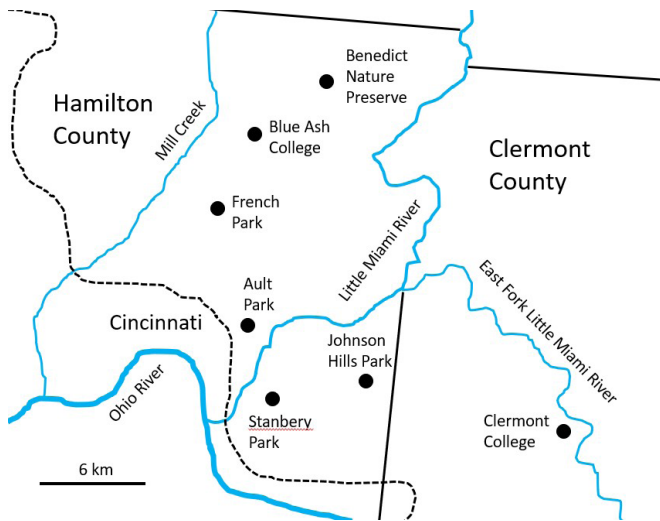


Fig. 1. Sampling locations (circles) for earthworms in Hamilton and Clermont Counties, Ohio, USA. Dashed line indicates approximate transition between Eastern Corn Belt Plains (northeast) and Interior Plateau (southwest) ecoregions.

Sampled locations included riparian zones and upland forest in natural areas of Hamilton and Clermont counties (Ohio, USA) in the Little Miami River and Mill Creek watersheds (Fig. 1). Sample sites were located at least 30 m away from patch edges. We preserved earthworms in ethanol following Chang et al. (2016). For molecular identification of randomly selected specimens, we sequenced the mitochondrial cytochrome c oxidase subunit 1 (COI) gene.

To isolate DNA, we removed 2–4 mm of tissue from the anterior ends of specimens and allowed it to dry. We then added 225 μ L of 50 mM NaOH to each sample and incubated at 95°C for 20 minutes. Samples were briefly centrifuged and immediately placed on ice. We added 25 μ L of 1 M Tris (pH 7.5) to each sample, and these were briefly vortexed and centrifuged again to ensure precipitation of debris. The supernatant was immediately used for PCR, and the remaining sample was stored at 4°C.

Primers were designed for partial amplification of COI based on Schult et al. (2016) with the following changes: primer sequences (sourced from Integrated DNA Technologies) were modified by addition of the M13(-21) sequencing primer sequence on the forward primer (5' TGT AAA ACG ACG GCC AGT TAY TCW ACW AAY CAY AAA GAY ATT GG 3') and the SP6 sequencing primer sequence on the reverse primer (5' ATT TAG GTG ACA CTA TAG TAK ACT TCT GGR TGM CCA AAR AAT CA 3') to facilitate direct sequencing of PCR products in either or both directions as needed. We used 1 μ L of sample DNA in a 25 μ L final reaction volume using DreamTaq and DreamTaq Buffer at manufacturer-recommended concentrations (Thermo Fisher Scientific). Reaction conditions were as follows: single initial denaturation at 95°C for 5 minutes; 40 cycles of 30 seconds at 95°C, 30 seconds at 40°C, and 1 minute at 72°C; followed by a final 7-minute extension at 72°C. The products were

electrophoresed through 0.8% agarose gel, and the amplicon was excised from the gel and extracted using a Promega Wizard Gel Purification kit. We quantified the purified samples and then sequencing was performed by Cincinnati Children's Hospital.

COI sequences were aligned and trimmed at each end to remove any ambiguity, leaving a consistent region of 603 bases for analysis. We compared our sequences to known sequences using BLASTN in NCBI at default settings. We confirmed identities using search results with at least 95% query cover, an E value of 0, and at least 95% identity.

RESULTS

Of the 58 COI sequences obtained, 90% consisted of non-native individuals. Forty belonged to nine non-native species in five genera of Lumbricidae (Table 1), and twelve belonged to non-native species in Megascolecidae and Moniligastridae (Table 2). The remaining 10% of sequences were native species in Acanthodrilidae and Sparganophilidae. Despite all specimens being collected from beneath surface cover objects, our earthworms were not all epigeic and represented a range of ecological types (Tables 1 and 2), although none were corticole.

All of our lumbricid species were previously documented in Ohio and the Cincinnati region except *Lumbricus castaneus*. Most of the lineages of these lumbricids have been previously reported from North America, with the exception of our specimen of the “new” lineage of *Octolasion tyrtaeum (lacteum)* (Table 1), which originates in Belarus (Shekhovtsov et al. 2014). Interestingly, within the L3 lineage of *Aporrectodea caliginosa (turgida)*, we found individuals of two distinct geographic origins: most shared close identity with specimens from France, but one (NCBI accession number PP118229) shared closest identity with a specimen from Kamchatka, Russia (KF471835).

Our specimens of megascolecids (Table 2) included all members of a three-species cryptic complex that has been documented in other parts of eastern North America: *Amyntas agrestis*, *A. tokioensis*, and *Metaphire (Amyntas) hilgendorfi* (Chang et al. 2018). *Amyntas tokioensis* has not been previously detected in Ohio or the Cincinnati region.

We identified two of our earthworms to Moniligastridae (Table 2), with closest similarity to specimens from Japan identified in NCBI as Moniligastridae sp. (LC703200) and *Drawida* sp. (AB592437). The closest known sequence identified to species was *Drawida koreana*, collected from Taiwan (KR047040), with other similar sequences to ours identified as *Drawida japonica*.

Our remaining COI sequences matched the native genera *Diplocardia* (Acanthodrilidae) and *Sparganophilus* (Sparganophilidae) (Table 2). Most of our *Diplocardia* had highest similarity to *Diplocardia caroliniana* from the southern USA (for example, HM386186 in NCBI). In contrast, one *Diplocardia* sequence was most similar

to *Diplocardia singularis* (HQ983626) from Oklahoma, USA. All of our *Diplocardia* specimens showed high similarity (> 98%) to *Diplocardia* collected by Ikeda et al. (2019) and provisionally designated as *Diplocardia* sp. 1 (for example, LC475530 and LC475573). Our sample contained one *Sparganophilus*, which was most similar to the species designated as *Sparganophilus* sp. 2 (for example, MK971750; Ikeda et al. 2019).

DISCUSSION

We detected many previously known non-native earthworms from Ohio and the Cincinnati region as well as several new introductions. However, we found very few native species. Most non-natives were lumbricids long established and widely distributed in Ohio (Olson 1928, 1933; Reynolds 2015). The only lumbricid not previously detected, *Lumbricus castaneus*,

Table 1. COI identities of lumbricid earthworms.

Species	Ecotype	Lineage ^a	Location ^b	Date	NCBI Accession Numbers
<i>Allolobophora chlorotica</i>	endogeic	L2	SP	Sep 2018	PP118223, PP118224
			FP	Sep 2017	PP118199
<i>Aporrectodea caliginosa (turgida)</i>	endogeic	L2 L3	BA	May 2019	PP118235
			BP	Sep 2018	PP118221
			JH	Nov 2018	PP118229
				Dec 2018	PP118230
			BA	May 2019	PP118236
				Jun 2019	PP118246, PP118247
			CC	Sep 2018	PP118231
	Oct 2018	PP118228			
<i>Aporrectodea rosea</i>	endogeic	L1/E1	BA	Jun 2019	PP118245
<i>Aporrectodea trapezoides</i>	endogeic	L2	BA	May 2017	PP118217
<i>Eiseniella tetraedra</i>	epigeic/ limicolous	--	AP	Aug 2017	PP118203
			BA	Jun 2017	PP118219
			FP	Aug 2017	PP118204
				Sep 2017	PP118200
			SP	Sep 2017	PP118211, PP118212
<i>Lumbricus castaneus</i>	epigeic/ endogeic	L1	BA	Apr 2017	PP118193, PP118194, PP118195, PP118196, PP118197, PP118198
				Jun 2019	PP118241, PP118243
<i>Lumbricus rubellus</i>	epigeic/ endogeic	L2/LA	BP	Sep 2018	PP118220, PP118225, PP118226, PP118227
			BA	Sep 2017	PP118216
			CC	Sep 2018	PP118232
			SP	Sep 2017	PP118209
				Sep 2018	PP118222
<i>Lumbricus terrestris</i>	anecic	--	BP	Sep 2018	PP118222
<i>Octolasion tyrtaeum (lacteum)</i>	endogeic	"new" (Belarus) "small"	BA	Jul 2017	PP118190
			FP	Aug 2017	PP118207
			SP	Sep 2017	PP118213, PP118214

^aLineage designations follow Shekhovtsov et al. (2014), Martinsson et al. (2015), and Porco et al. (2018).

^bLocations are Ault Park (AP), Harris M. Benedict Nature Preserve (BP), French Park (FP), W. M. Johnson Hills Park (JH), Stanbery Park (SP), University of Cincinnati Blue Ash College (BA), University of Cincinnati Clermont College (CC).

Table 2. COI identities of non-lumbricid earthworms.

Family	Species ^a	Ecotype	Location ^b	Date	NCBI Accession Numbers
Acanthodrilidae	<i>Diplocardia caroliniana</i> (sp. 1)	endogeic	BA	May 2019	PP118233, PP118237, PP118238
				Jun 2019	PP118244
	<i>Diplocardia singularis</i> (sp. 1)	endogeic	BA	May 2019	PP118240
Megascolecidae	<i>Amyntas agrestis</i>	epigeic/ endogeic	BA	May 2017	PP118218
	<i>Amyntas tokioensis</i>	epigeic/ endogeic	AP	Aug 2017	PP118205, PP118201, PP118202
			BA	Jun 2017	PP118192
				Jul 2017	PP118215
		Sep 2017	PP118210		
	<i>Metaphire (Amyntas) hilgendorfi</i>	epigeic/ endogeic	BA	Jun 2017	PP118191
BA			Sep 2017	PP118208	
			Jun 2019	PP118242	
Moniligastridae	<i>Drawida</i> sp.	epigeic	BA	May 2019	PP118234, PP118239
Sparganophilidae	<i>Sparganophilus</i> sp. 2	limicolous	BA	Sep 2017	PP118206

^a*Diplocardia* and *Sparganophilus* provisional species designations are from Ikeda et al. (2019).

^bLocation abbreviations are as in Table 1.

has possibly been overlooked, as it is present in surrounding states (Reynolds 2018). The COI lineages of our lumbricid specimens (Table 1) have mostly European native distributions and are known to be spreading widely around the world (Martinsson et al. 2015; Porco et al. 2018).

The second largest group in our sample was non-native megascolecids. Various species have been recorded in Ohio, Indiana, and Kentucky since the 1950s, and *Amyntas agrestis* and *Metaphire hilgendorfi* have been established in northern Ohio since at least 2010 (Reynolds 2018). We detected these two species along with *Amyntas tokioensis* (Table 2), and this was the most common species, which is consistent with other observations (Chang et al. 2018). All are part of an invasion complex and are typically encountered together.

Additionally, we detected two *Drawida* (Moniligastridae). These are part of a species complex that includes *Drawida koreana* and *Drawida japonica*, both native to eastern Asia and not previously documented in continental North America (Blakemore 2009; Zhang et al. 2012), although there is one unconfirmed report of *D. japonica* in Kansas (Blakemore et al. 2014). The ecology of these *Drawida* is not well studied, although *D. japonica* is epigeic in forests, ditches, and pond perimeter soils (Zhang et al. 2012). Our specimens were collected from beneath logs in two different locations of upland areas of the same woodlot and may represent a recent introduction or a very localized population.

Despite the overwhelming presence of non-native

earthworms in our sample, native species of *Diplocardia* and *Sparganophilus* persist. Our *Diplocardia* sequences were most similar to reference sequences of two closely related species: *D. singularis* (known from Ohio) and *D. caroliniana* (not known from Ohio). However, our sequences match only one widely distributed molecular lineage that Ikeda et al. (2019) designate as *Diplocardia* sp. 1. It is currently unclear how these molecular species designations correspond to traditional morphological species. For *Sparganophilus*, only *S. eiseni (tamesis)* is previously known from Ohio, a species probably equivalent to *Sparganophilus* sp. 1 of Ikeda et al. (2019). The COI sequence of our specimen instead aligns with *Sparganophilus* sp. 2 (Ikeda et al. 2019), indicating the presence of an unreported *Sparganophilus* in Ohio.

One native group notably absent from our sample is lumbricids in the genera *Bimastos* and *Eisenoides*. Six species are known from the Cincinnati region and have scattered distributions through Ohio (Reynolds 1994, 2008, 2015). Consequently, our sample may not have been large enough to detect these species. However, it is also possible that they have been negatively impacted by competition with non-native earthworms or by anthropogenic disturbances that have reduced their habitat.

Our work demonstrates that sampling of earthworms from beneath cover objects coupled with molecular identification can reveal important clues about the status of native and introduced earthworm species. Of great concern is that despite collecting from relatively undisturbed non-edge habitat in natural areas,

the majority of the earthworms identified were non-native species. Given the potential negative ecological impacts of non-native earthworms (Hendrix et al. 2008), more work is needed to further determine species presence and relative abundance. Such work can help identify trends in distributions relative to historical records, many of which have not been revisited in Ohio in almost a century. In particular, molecular identification can also detect cryptic earthworm species and lineages, shed light on their geographical origins, and help wildlife managers better predict what impact new introductions will have on local ecosystems.

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