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# A NEW SPECIES OF *MONOCYSTIS* (APICOMPLEXA: GREGARINA: MONOCYSTIDAE) FROM THE ASIAN INVASIVE EARTHWORM *AMYNTHAS AGRESTIS* (MEGASCOLECIDAE), WITH AN IMPROVED STANDARD FOR *MONOCYSTIS* SPECIES DESCRIPTIONS

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**KEY WORDS**

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*Amynthas*
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*Monocystis perplexa*
Species Descriptions
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*18S rRNA* Gene
Invasive Earthworms
Parasite Life Stages

**ABSTRACT**

*Monocystis perplexa* n. sp., a parasite of an important invasive Japanese earthworm in North America, *Amynthas agrestis*, is described from a site in Vermont. An improved standard for *Monocystis* species descriptions is proposed including a standard nomenclature to reduce synonymies, a standard set of biometrics and shape descriptions for living cells, and a DNA genomic sequence for the *18S rRNA* (~1,700 base pairs). Comparing morphologies of *Monocystis* parasites in sympatric earthworm species indicates that *M. perplexa* is specific to *A. agrestis* in the study region. Also, polymerase chain reaction primers specific to *M. perplexa* amplified samples of *A. agrestis* earthworms taken from several sites in Japan. This suggests the parasite entered North America from Japan, the origin of the invasive *Amynthas* earthworm, and thus *M. perplexa* would be the first *Monocystis* described from the diverse Japanese *Amynthas* earthworms and the first from East Asia. *Monocystis perplexa* was found in every population of *A. agrestis* surveyed in Vermont, always reaching 100% prevalence by late summer (the host has an annual life cycle in Vermont). The *18S* gene sequence differed from that of *Monocystis agilis* from the sympatric earthworm *Lumbricus terrestris* (the only other sequence available for *Monocystis*), and a genetic similarity tree places them closest among other gregarines. Many of the 95 described species of *Monocystis* are very similar in morphology (based on species descriptions), so the *18S* gene can act as a barcode for *Monocystis* species and thus will help to eliminate both synonymies and reveal cryptic species.

The gregarines are a clade of early-branching apicomplexan parasites that infect a broad diversity of invertebrate animals from marine, freshwater, and terrestrial environments (Desportes and Schrével, 2013). Cavalier-Smith (2014), using molecular, cell structural, and life cycle data, found the classical gregarines are polyphyletic and erected a new class, Gregarinomorphea, to hold most of the overall diversity (here we term "gregarines"). Like other apicomplexans, the gregarine life cycle includes sexual reproduction to produce the transmission stage (oocysts) but differs in lacking merogony (asexual replication) in most species. The genus *Monocystis* (Monocystidae) can stand as an exemplar in many positive ways for the gregarines. Fully 95 described species are known from earthworm hosts from Europe, North and South America, West Africa, and South and Southeast Asia (Levine, 1977; Desportes and Schrével, 2013). Because the parasite often reaches 100% prevalence in earthworm populations (Meier, 1956; Miles, 1962), and all the life stages can readily be seen in live preparations, *Monocystis* has long been used as a representative of the gregarines in parasitology courses (Grove, 1923; Sheridan, 1986). Unfortunately, *Monocystis* can also stand as an exemplar of taxonomic difficulty for the gregarines. From the earliest work on *Monocystis* species (von Stein, 1848; Berlin, 1924), synonymies were common (Levine, 1977). Eleven *Monocystis* species have been described from the common nightcrawler *Lumbricus terrestris*; this seems an unlikely high diversity of valid taxa in a single earthworm species (Levine, 1988; Segun, 1971). Levine (1977) in his review of the Monocystidae notes that many species have similar morphology and wonders if described species are valid. Wenyon (1926, p. 1147) states that described species of *Monocystis* "differ from one another in details only," and Rees (1961) and Segun (1968) draw a similar conclusion. Indeed, *Monocystis* species are often barely recognizable based on their descriptions, except for host taxon (Levine, 1977). The counter problem is also likely the presence of cryptic species. For example, *Monocystis agilis* of the familiar *L. terrestris* suspiciously has been

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described from 6 earthworm species, some not closely related and from distant geographic sites (Levine, 1977). Certainly, any understanding of *Monocystis* diversity, evolutionary relationships, and biogeography requires improved species descriptions.

Here we describe a new species of *Monocystis* from an invasive Japanese earthworm *Amynthas agrestis* (Megascolecidae) now causing severe environmental disruption in many areas of North America (Görres and Melnichuk, 2012). *Amynthas agrestis* was introduced into the United States in the 1860s and has since spread throughout the continental United States (Gates, 1954; Snyder et al., 2011). Within the last decade, ongoing surveys began identifying the presence of *A. agrestis* in Vermont and these earthworms are now found throughout the state (Görres and Melnichuk, 2012; Reynolds et al., 2015; Görres et al., 2018; Nouri-Aiin and Görres, 2019). Almost certainly, *A. agrestis* will continue to spread and researchers studying invasive earthworms will readily detect the parasite because it often reaches high density in the earthworm's seminal vesicles. We follow a protocol that should improve the description of *Monocystis* species.

First, the standard terminology for characters of Levine (1971) and the biometric and detailed set of morphological shapes assembled by Clopton (2004) are followed. Comparison across species is fostered by high-quality photographs of living cells. Living *Monocystis* parasites have long been known to be morphologically plastic (Braun and Lühe, 1910), and fixed and stained preparations often result in distorted and shrunken parasites that make identifying diagnostic characters difficult (E. Keller and J. Schall, pers. obs.; and see Bosanquet, 1894). Therefore, live preparations used with light microscopy are preferred.

Second, a survey of the parasites in the community of sympatric potential hosts provides insight into *Monocystis* host specificity. For example, in North America typically 6 species of earthworms are found sympatric (Tiunov et al., 2006), and earthworms could readily consume the oocyst transmission stages from *Monocystis* shed by other earthworm species. Although gregarines, in general, are assumed to be primarily host species-specific (Clopton et al., 1992), host specificity of gregarines has not been well studied (Clopton, 2009), especially with confusion in species identification. Also, the use of polymerase chain reaction (PCR) primers specific to the new *Monocystis* species allowed a survey for the parasite in *A. agrestis* from its origin in Japan.

Finally, a dearth of molecular data for *Monocystis*, and indeed gregarines in general, impedes the recognition of new species. Perkins et al. (2011) note that a modern diagnosis of protist parasites requires both standardized morphological data and also gene sequences. Both nuclear and mitochondrial genes have been used as barcodes for eukaryotes (Hebert et al., 2002), and the cytochrome oxidase I (*COI*) gene as a molecular barcode has been used successfully for some apicomplexan parasites, such as the coccidians (Ogedengbe et al., 2011). However, the *COI* gene appears to be absent in gregarines (Putignani et al., 2004; Templeton et al., 2010). While more often used in resolving deep-branching phylogenies, the gene coding for the *18S SSU rRNA* has also had success as a barcoding gene for apicomplexans (Morrison and Ellis, 1997). The *18S* rRNA gene is useful for barcoding because it harbors both conserved and variable regions and is present in multiple copies in the genome (Renoux et al., 2017).

*Monocystis* probably has a world-wide distribution wherever earthworms are present, and every earthworm species seems to harbor one or more specialized species. Thus, *Monocystis* offers many questions in speciation, biogeography, and ecology, provided species are unambiguously described. We describe a *Monocystis* species from an invasive earthworm in Vermont and provide evidence that the parasite arrived when the earthworms were introduced from Japan, thus extending the known range of the genus to East Asia. We implement an improved protocol for the description of *Monocystis* species. Any understanding of the taxonomy and relationships of the large number of *Monocystis* species described from earthworm hosts across 5 continents requires such a standard method of species description.

## MATERIALS AND METHODS

In Vermont, *A. agrestis* earthworms reside in the top 10 cm of soils, often sympatrically with the congeneric *Amynthas tokioensis* and *Amynthas hilgendorfi*. Earthworms from the type locality were collected by manually sifting through the top 10 cm of soil between July and October 2017. The new *Monocystis* species was studied in 19 *A. agrestis* earthworms from the type locality. Additional *A. agrestis* earthworms were collected from the type locality and 2 other local Vermont sites to determine the prevalence and distribution of the parasite. There is no genetic structure between the Vermont populations of *A. agrestis* suggesting they may be from a single source population (Keller et al., 2017). All adult earthworms sampled were confirmed to be *A. agrestis* using a morphological key of pheretimoid earthworms (Chang et al., 2016).

All earthworms were killed by submersion in 50% ethanol and then washed in $dH_2O$. The parasites are primarily found in the earthworm seminal vesicles (the organ for the maturation of self-sperm), so these were dissected and disrupted with 1× earthworm Ringer's solution and 2 µl of the live preparations placed under a coverslip and examined with a light microscope. Live preparations were preferred to fixed preparations because we observed dramatic distortion, shrinking, and loss of detail in cells caused by the fixing and staining. Further, monocystids often distort their shape while moving, so observing these morphological changes in live specimens can reduce false identification of new species based on morphological differences in fixed specimens. Measurements and photographs of gamonts, gametocysts, and oocysts were taken using a Moticam 1000 1.3MP Live Resolution (Motic, Richmond, British Columbia, Canada) microscope camera and Motic Image Plus 2.0.11 computer program. Nineteen *A. agrestis* used for the species description from the Green Mountain Audubon Center were dissected and trophozoites, gamonts, gametocysts, and oocysts were measured according to the guidelines published by Clopton (2004) and photographed.

Pure parasite DNA was prepared by concentration, cleaning, and disruption of the gametocyst stages. Briefly, earthworm seminal vesicles were manually disrupted in earthworm Ringer's solution to allow the gametocysts to fall out and be removed. These were washed with the Ringer's, then vortexed with a small amount of 0.5 mm zirconia beads for 1 min to break them open and for oocysts to emerge. After washing in saline, the oocysts were cleaned with 10% sodium hypochlorite for 10 min, washed, and vortexed with the beads at 4,800 oscillations/minute (Mini Beadbeater, Biospec, Bartlesville, Oklahoma) in lysis buffer from the DNeasy blood and tissue kit (Qiagen, Valencia, California), and then the DNA was extracted using the kit and manufacturer's protocol.

The gene coding for the *18S SSU rRNA* was amplified in a single fragment using universal eukaryotic primers Euk18F 5′-

CGA ATT CAA CCT GGT TGA TCC TGC CAG T-3′ and Euk18R 5′-CCG GAT CCT GAT CCT TCT GCA GGT TCA CCT AC-3′ previously described by Leander et al. (2003). PCR was run using the TopTaq PCR master mix kit (Qiagen) and manufacturer's instructions. The PCR conditions were as follows: Initial denaturation: 95 C 2 min, followed by 35 cycles of 92 C/45 sec, 60 C/45 sec, and 72 C/1.5 min, with a final extension at 72 C for 5 min. The PCR yielded a single amplicon of ~1,700 base pairs (bp). The amplicons were Sanger sequenced at the University of Vermont genetic analysis core facility.

Because *A. agrestis* earthworms are often found in sympatry with local and invasive earthworms of Vermont, the parasites infecting the seminal vesicles of 8 sympatric earthworm species were examined. All earthworm samples were collected within Chittenden County, Vermont and the dissection and live preparation of slides of the seminal vesicle infections utilized the methods described above. The following local earthworm species were sampled and examined for the presence of *M. perplexa*: *Aporrectodea turgida* (n = 3), *Amynthas tokioensis* (n = 77), *Amynthas hilgendorfi* (n = 15), *Lumbricus terrestris* (n = 5), *Lumbricus festivus* (n = 1), *Lumbricus rubellus* (n = 3), *Octolaseon cyaneum* (n = 5), and *Dendrobaena octaedra* (n = 1). Apparent morphology of the observed parasite life stages for each host species was compared to that of the new species to determine if it was present in sympatric host species.

*Amynthas agrestis* is native to Japan, so the origin of *M. perplexa* was sought in earthworms collected from several sites on Honshu Island. Seminal vesicles (n = 28 earthworms) were preserved in lysis buffer from the DNeasy kit. Species-specific primers were developed by aligning the *18S* sequences from *M. perplexa* and *Monocystis agilis* (below) and searching for regions that differed substantially. The primers used were MP250F 5′-GGT GAT CCA TAA TAA TGT CGC AGA-3′ and MP277R 5′-CGG TAG GAC AAT ACC CGA CTG-3′ which gave a product of 107 bp. The PCR conditions were as follows: Initial denaturation: 94 C 3 min, followed by 40 cycles of 94 C/50 sec, 59 C/30 sec, and 72 C/1 min, with a final extension at 72 C for 4 min. The PCR yielded a single amplicon of ~100 bp. Again, the PCR was run using the TopTaq PCR master mix kit.

A genetic similarity tree was constructed to compare *M. perplexa* and *M. agilis* (the only other *Monocystis* species for which *18S SSU* gene sequence is available) with other gregarines selected from 6 families taken from GenBank (see Results). The sequences were aligned and a neighbor-joining tree constructed with nodes challenged with 1,000 bootstraps using ClustalX 2.1 (Larkin et al., 2007).

## DESCRIPTION

### *Monocystis perplexa* n. sp.

(Fig. 1)

Phylum Apicomplexa Levine, 1988
Class Gregarinomorphea Cavalier-Smith, 2014
Order Arthrogregarida Cavalier-Smith, 2014
Family Monocystidae Bütschli, 1882
Genus *Monocystis* von Stein, 1848

*Diagnosis:* Based on 19 *Amynthas agrestis* earthworms examined from the type locality with live wet mounts under coverslip using life stages defined by Levine (1971). Trophozoites vary in shape as they move and size as they grow, but generally orbicular (Clopton E3), mature gamonts (n = 346) when alive also can vary in form as they move, but generally are rhomboid in shape (Clopton C5) with length, the distance along vertical axis of symmetry (L ± SD) 49.06 ± 12.43 μm, width, the distance along horizontal axis of symmetry (W ± SD) 27.20 ± 7.26 μm, and with a round sucker-like structure with a retractile central tentacle located mesolaterally (Fig. 1A). Orbicular (Clopton E3) nuclei (n = 316) situated typically in the center of gamonts, diameter (D ± SD) 10.33 ± 2.54 μm. Trophozoites and gamonts are solitary until syzygy when gamonts attach along their long axis (n = 75 gamonts observed in syzygy). Isogametes seen in mating gamonts indicate lack of anisogamy (Fig. 1B). Mature gametocysts (n = 150) are orbicular in shape (Clopton E3) with D 120.74 ± 16.43 μm (Fig. 1C). Oocysts (n = 170) are fusiform in shape (Clopton C4) with L 15.37 ± 0.74 μm and W 5.65 ± 0.53 μm (Fig. 1D).

### Taxonomic summary

*Type and known host: Amynthas agrestis* Kinberg 1867 (Megascolecidae).

*Type locality:* The Green Mountain Audubon Center, Huntington, Vermont (190 m elevation, 44°20′48.4″N, 72°59′46.4″W).

*Other localities:* UVM Horticultural and Research Farm, South Burlington, Vermont (44°25′53.4″N, 73°11′57.2″W); UVM Centennial Woods, Burlington, Vermont (44°28′32.5″N, 73°11′13.5″W). The 3 Vermont sites are all within 19 km distance.

*Site of infection:* Infections are found primarily in seminal vesicles of *Amynthas agrestis* and occasionally seen in the body coelomic fluid.

*Prevalence of infection:* Overall prevalence in *Amynthas agrestis* was 603 of 603 (100%) by mid-summer to time of seasonal host death in mid-autumn.

*Specimens deposited:* Holotype (United States National Museum [USNM] 1618942) and paratypes (USNM 1618943, USNM 1618944) deposited at the Smithsonian Museum of Natural History, and additional paratypes (AU24, AU5) deposited at University of Vermont Zadock Thompson Zoological Collections, University of Vermont Natural History Museum.

*ZooBank registration:* urn:lsid:zoobank.org:act:78920EC4-AB15-4B79-86DB-EB0F6EFDC7A2.

*Etymology: Monocystis perplexa*, from the Latin word *perplexa* meaning intricate and complicated. *Monocystis perplexa* is an appropriate name that refers to the protean morphology of this parasite's trophozoites and even gamonts, as they can change in shape while moving. Further, significant aspects of the life history and life cycle of *Monocystis* spp. remain unresolved and perplexing.

*Molecular sequences/DNA Sequences: 18S* small subunit ribosomal RNA gene (1,665 bp, GenBank MT622497).

### Remarks

This is the first species of *Monocystis* described from *Amynthas agrestis*, a highly invasive earthworm introduced from Japan (Keller et al., 2017). The parasite is likely to have traveled with its host's origin because parasite species-specific PCR primers achieved amplification for all *A. agrestis* collected from Japan. Thus, *M. perplexa* is likely to be the first *Monocystis* described from Japan (albeit indirectly).

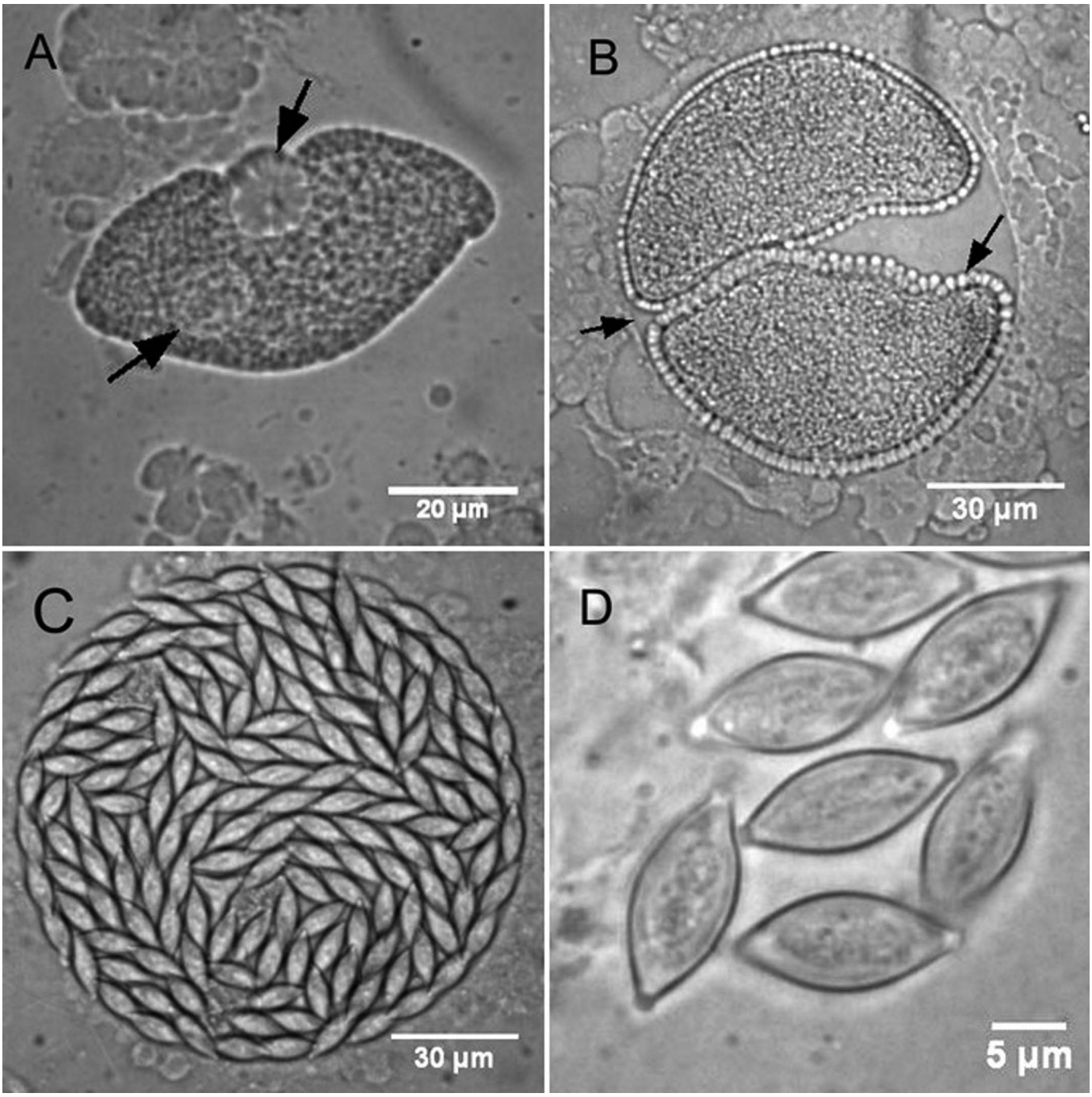


**Figure 1.** Life stages of *Monocystis perplexa* n. sp. in the earthworm *Amynthas agrestis*. (**A**) Gamont with arrows pointing to the nucleus toward anterior end and a sucker-like structure of unknown function protruding from mesolateral cell margin. (**B**) Pair of gamonts in syzygy, the entry into the sexual cycle; the arrows point to gametes produced by each cell (isogametes meaning no sexual dimorphism and thus no gender). (**C**) Mature gametocyst containing oocysts, the transmission stage deposited in the soil. (**D**) Oocysts.

In concord with all parasites of the family Monocystidae (Levine, 1977, 1988), *M. perplexa* is characterized by solitary gamonts with little anterior differentiation and fusiform oocysts. An examination of earthworms of 8 species sympatric in the region where *A. agrestis* has been surveyed found all except *A. hilgendorfi* were infected with an apparent monocystid species but, based on morphology and biometric measurements, *M. perplexa* is found only in *A. agrestis* earthworm hosts due to the distinct morphological differences among parasites in the other earthworms. For example, none of the other monocystids exhibit the round, sucker-like central lateral structure seen in *M. perplexa*, several others (such as in *A. tokioensis*, *L. terrestris, and L. rubellus*) produce gametocytes with apparently thousands of oocysts, and in *Dendrobaena octaedra* the gametocyts produce far fewer oocysts arranged primarily around the periphery. Gamonts in syzygy also differ as in *Aporrectodea turgida* in which the cells attach at their terminal ends and in *L. rubellus* in which the gamonts are spherical rather than in *M. perplexa* with its rhomboid gamonts that attach along their long axis. Noteworthy is that parasites were seen in all these earthworm species despite rather small sample sizes, and that the parasites often did not match species descriptions of any monocystid and thus may represent unknown taxa.

Further, *M. perplexa* appears to be widespread among local *A. agrestis* populations based on the presence of *M. perplexa* at 2 sites in addition to the type location. *Monocystis perplexa* can also be easily distinguished from other described *Monocystis* species found in *Amynthas* and *Metaphire* earthworms based on gamont, gametocyst, and oocyst morphology. The 2 most similar *Monocystis* species to *M. perplexa* are *Monocystis amynthae* from *Amynthas hawayanus* and *Monocystis metaphirae* from *Metaphire houlleti* and are compared below.

The gamonts of *Monocystis amynthae* (Bandyopadhyay et al., 2006a) and *Metaphire metaphirae* (Bandyopadhyay et al., 2006b) are longer, wider, and of different morphology than *M. perplexa* gamonts. *Monocystis perplexa* gamonts are rhomboid, with tapered anterior and posterior ends, smooth edges, and no marked mucron, whereas *M. amynthae* and *M. metaphirae* gamonts have broad anterior ends, narrow posterior ends, and anterior mucrons. All 3 *Monocystis* species contain irregularly shaped paraglycogen granules uniformly located throughout the gamont cytoplasm; however, *M. amynthae* gamonts are uniquely constricted between the anterior and posterior ends. At the center of *M. perplexa* gamonts and the anterior end of *M. amynthae* gamonts, both species contain a round, cup-like depression in the center to anterior end with unknown function. Further distinctions between *M. perplexa, M. amynthae*, and *M. metaphirae* come from morphometric differences in gametocysts; *M. perplexa* gametocysts are nearly twice as large as those of *M. amynthae* and *M. metaphirae*; however, this may be due to differences in slide preparations. When preparing slides without a coverslip and with methodology similar to Bandyopadhyay (2006a, 2006b), *M. perplexa* gametocysts remain larger in diameter (75.02 μm) than *M. amynthae* (58 μm) but smaller than *M. metaphirae* (93 μm). Additionally, *M. perplexa* oocysts are longer (15.4 μm) and have a larger ratio of oocyst length to width (2.7) than *M. amynthae* (oocyst length 10.5 μm; ratio 1.9) and *M. metaphirae* (oocyst length 9.0 μm; ratio 1.6).

Perhaps the most useful distinction between *Monocystis* species is the molecular data of a taxonomically informative gene, the *18S SSU rRNA* gene. For example, *M. perplexa* can be easily distinguished using the *18S rRNA* gene sequences from the only other *Monocystis* species available on GenBank, *M. agilis* (AF457127.1); the *18S* sequence of *M. agilis* shares 83.0% identity with *M. perplexa*, which highlights the usefulness of the *18S* sequences as a barcoding gene for the gregarines. A genetic similarity neighbor-joining tree for a variety of gregarines (Fig. 2) places the 2 *Monocystis* species together. As additional *18S* sequences become available for the 95 known and other *Monocystis* species found world-wide, this gene should allow a phylogeny to be recovered to determine if the morphological *Monocystis* taxa are indeed a monophyletic group.

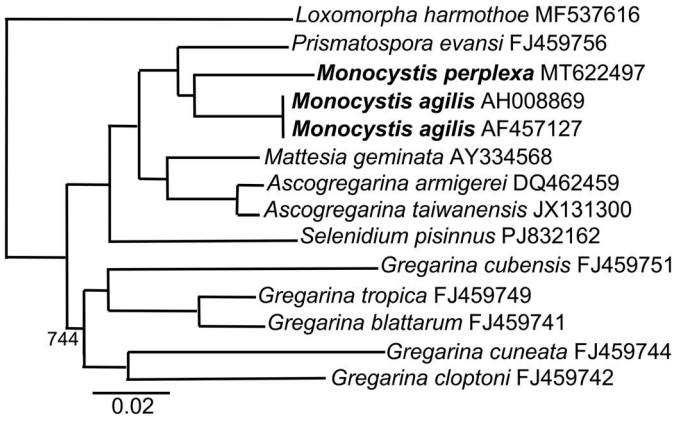


**Figure 2.** A genetic similarity (neighbor-joining) tree for the *SSU 18S* ribosomal RNA gene for gregarines of several families. Species names and GenBank accession numbers given on the tree. Two *Monocystis* species are shown in bold. All nodes supported by >95% of 1,000 bootstraps, except for one indicated node. The new species, *Monocystis perplexa*, is shown to be most similar to *M. agilis*, the only other *Monocystis* species for which a sequence is available for the *18S* gene.

## DISCUSSION

Although Asian *Amynthas* and related earthworm genera have been known in North America since 1867, they have only in the past 2 decades become important invasives, with 16 species now present across the United States (Chang et al., 2016). *Monocystis* species have been described from Indian *Amynthas* species (review above in Remarks), but *M. perplexa* is the first known from *Amynthas* originating from Japan and the first described from the invasive populations of *Amynthas* in North America. Thus, the possible distribution of *Monocystis* is extended to East Asia. Blakemore (2003) records 51 pheretimoid earthworm species in Japan (*Amynthas* and related genera), so if most species of earthworms harbor their specialized *Monocystis* species, a full account of the Japanese *Monocystis* species could substantially increase the known taxa in the genus.

One goal of this species description was to redefine and provide an example of what should be included in future *Monocystis* species descriptions. First, the standardized nomenclature and biometrics given by Clopton (2004) will reduce synonymy in terminology. Second, in cases where the host species of interest inhabits a community with other potential hosts of gregarines (e.g., earthworm communities), the infection status of the sympatric species should be investigated to determine if the

*Monocystis* species are shared between host species; this can provide important data regarding the host specificity of the gregarines. Last, molecular data should be provided with species descriptions to diagnose species that are very similar in morphology as well as to aid in the production of robust phylogenies of the parasites in subsequent studies. That is, does a geographic signal obtain in the phylogeny of the diverse and widespread *Monocystis* of earthworms?

Implementing the above suggestions in the species description of *Monocystis perplexa* provided important insight into the diversity, specificity, and distribution of *Monocystis* species. Notably, *M. perplexa* and other *Monocystis* spp. were found to be host species-specific, with each species of earthworm appearing to have its own morphologically distinct species of monocystid. *Monocystis perplexa* infections were found in all *A. agrestis* earthworms sampled but, based on morphology, *M. perplexa* was not found in any other local earthworm species, including the congeneric *A. tokioensis* which has its own distinctive monocystid. Beyond providing evidence for host specificity of *Monocystis*, the failure to identify *M. perplexa* in sympatric host species and evidence for the presence of *M. perplexa* in the host collected in Japan suggests that *A. agrestis* earthworms retained their native infections when invading a new soil community. Retaining the parasite has not hindered the invasive success of the earthworm host.

Last, the use of the gene coding for the *18S SSU rRNA* as a barcode is promising. The *18S* sequences obtained from *M. perplexa* is here proposed diagnostic and we found that common sequence in the parasites at all 3 locations. The *18S* gene sequenced from *M. perplexa* clustered with *M. agilis*, as was expected based on the diagnostic characteristics of the genus.

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