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APOLOCYSTIS BOSANQUETI N. SP. (APICOMPLEXA: EUGREGARINORIDA) FROM THE INVASIVE EARTHWORM *AMYNTHAS AGRESTIS* (ANNELIDA: MEGASCOLECIDAE), WITH SIGNIFICANCE FOR THE MONOPHYLY OF THE FAMILY MONOCYSTIDAE

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ABSTRACT

Apolocystis bosanqueti n. sp., a parasite of an important invasive earthworm in North America, *Amynthas agrestis*, is described from a site in northern Vermont. The earthworm host follows an annual life cycle in Vermont, so the entire life cycle of the parasite can be observed in 7 mo. In spring, the parasites were first seen in juvenile worms as paired gamonts (suggesting precocious association). These paired gamonts mature into gametocytes that form an opaque structure, with a thick gelatinous envelope (epicyst), that becomes full of zygotes. The resulting gametocyst becomes packed with $\sim 10^5$ fusiform oocysts. The mature orbicular gametocysts are large ($\sim$1 mm in diameter) and visible to the naked eye through the body wall of the host's anterior segments. The new species most resembles *Apolocystis herculea* described from many lumbricid earthworm species in Europe but differs from that parasite because *Ap. herculea* infects the intestinal wall in the posterior of the host rather than the anterior segments. A survey of 9 other earthworm species sympatric with *Am. agrestis* revealed that only *Amynthas tokioensis*, also an invasive species, was infected with *Ap. bosanqueti*, albeit much less commonly. Diagnosis for the family Monocystidae is problematic because cardinal characters are lacking, and the commonly cited character, a trophozoite with no anterior differentiation, is violated in most genera placed in the family. For the first time, a molecular phylogeny is presented that includes 3 genera of monocystids with diverse cell morphology (including the new species) and supports the monophyly of the family. The only morphological character that may be used to diagnose the Monocystidae is the morphology of oocysts, which are fusiform with extended terminal tips. A comparison of oocysts from 7 parasites recovered from local earthworms, including from 3 monocystid species in the phylogeny, confirms the utility of this diagnostic trait. The 2 hosts of the new species were most likely introduced from Japan, so the range of *Apolocystis* likely extends into East Asia.

Gregarines are protist parasites of the phylum Apicomplexa that are notable for their great diversity (likely millions of species), broad host range across most invertebrate groups, and often large cell size and morphological complexity (Desportes and Schrével, 2013; Rueckert et al., 2019). The life cycle is complex, with initial feeding stages (trophozoites) that develop into the mating form (gamonts) that pair as gametocytes within a developing gametocyst where they produce gametes, zygotes, and finally the transmission stages, or oocysts. Morphologically gregarines fall broadly into cells divided into compartments (septate) and those without such division (aseptate). Early investigators thought these large complex cells, especially the septate forms, were multicellular larvae of parasitic helminths (Cavolini, 1787; Dufour, 1828; Leidy, 1853). Dufour (1828) coined the term gregarine for the complex cells he observed in insects. The cells were often in groups, even joined, suggesting the name (gregarine = "herd"). Within a few years advances in microscope optics, such as correction for spherical and chromatic aberration, allowed detailed examination of gregarine cells. Henle (1845), Stein (1848), and especially Kölliker (1848) distinguished the aseptate from septate species, precocious from late association of gamonts, formation of the gametocyst, and finally oocysts. Kölliker (1848) concluded that the gregarines are single cells with elaborate structures and undergo a complex life history with changes in morphology as they fed, finally joining to produce the

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transmission stage cysts. The idea that single-celled organisms can have such developmental stages in their life history deserves note as a major finding in parasitology and, indeed, all of biology. These pioneering researchers made these discoveries because they recognized the value of the aseptate forms in earthworm hosts to follow this life cycle, and Stein (1848) erected the genera *Monocystis* and *Zygocystis* to highlight late vs. early association of the gamonts in the aseptate forms in earthworm hosts. The family Monocystidae (Bütschli, 1882) was later erected to include *Monocystis* and *Zygocystis* and other aseptate parasites and now contains 24 genera and more than 180 described species (Levine, 1988; Desportes and Schrével, 2013).

Despite this important role of monocystid parasites in the history of parasitology, diagnosis of the family Monocystidae is still problematic. Cardinal characters are given by Levine (1977, 1988) as "Gamonts spherical to cylindrical, with anterior end little differentiated if at all; oocysts biconical or navicular; mostly coelomic; the great majority parasites of oligochetes." Other authors concur with this diagnosis (Segun, 1968; Clopton, 2000; Desportes and Schrével, 2013). However, the genera placed into Monocystidae often have complex structures at the anterior end; fully 14 genera have suckers, funnels, trunks, or filaments. For example, *Stomatocystis goerresi* has an elaborate funnel at the anterior end that can be larger than the cell body (Schall, 2021). Thus, the only clear, uniform, morphological traits that may diagnose the family are the aseptate cell structure and the form of the oocyst, which has been described as biconical, fusiform, or navicular (boat-shaped). Clopton (2009) notes that the lack of clade-level cardinal characters to diagnose families is a common problem for gregarines. Bhatia (1930) sought to resolve the issue by dividing the monocystids into several families based on the morphology of the trophozoite, for example, Stomatocystidae for cells with anterior funnel or mouth-like structures. An obvious resolution to test the monophyly of the Monocystidae would be a well-supported molecular phylogeny, but phylogenetic studies of gregarines have included only a single species of monocystid, *Monocystis agilis*, the type species for the genus described by Stein (1848) (Leander et al., 2003a, 2003b; Cavalier-Smith, 2014; Simdyanov et al., 2017).

Also problematic is the placement of monocystid species into genera (Rees, 1962; Segun, 1968). An example is the striking parasite of earthworms described by Bosanquet (1894). This gregarine produces large gametocysts that can be seen through the body wall of the earthworm host as white spheres up to 2 mm in diameter. Bosanquet (1894) described the gamonts as spheres without polar differentiation seen as pairs that form the large gametocyst. He placed the parasite into *Monocystis* (*Monocystis herculea*) without explanation. Meier (1956) moved the species to the genus *Apolocystis* erected by Cognetti de Martiis (1923) to include species with a spherical gamont, but Cognetti de Martiis explicitly excluded *M. herculea* from the new taxon. Cognetti de Martiis also could not determine if *Apolocystis* parasites produce the fusiform oocysts expected for a monocystid. Segun (1968) gives this tangle as an example of the "vague" definition of monocystid genera. Since then reviews of the Monocystidae have maintained Bosanquet's parasite in *Apolocystis* (Levine, 1988; Kundu and Bandyopadhyay, 2019). *Apolocystis herculea* has now been described from 15 earthworm species from locations across Europe (review in Pizl, 1989). This wide host and geographic range suggest there may be more than a single species of parasite.

We confront these issues with the description of a new species of parasite similar to *Ap. herculea* that infects an important invasive earthworm introduced from Japan into North America, *Amynthas agrestis*. This earthworm was first reported in North America in 1939, but only in recent decades has it become a significant invasive pest with disruption of soils and soil communities across the continental United States (Nouri-Aiin and Görres, 2019). We follow the protocol for species description of monocystids given by Keller and Schall (2020) and Schall (2021) that uses the terminology for life stages of Levine (1971), the set of morphological shapes listed by Clopton (2004), the timing of gamont association and features of syzygy recommended by Clopton (2009), measurements of all life cycle stages of live cells, and photographs of live parasites. Also, we follow the proposal of Clopton (2009) to include information on the structure of the gametocyst envelope or epicyst and the possible process of oocyst liberation or dehiscence. Live cells were chosen for study and measurement because monocystids are noted as morphologically protean during movement (Braun and Lühe, 1910; Rees, 1962; Keller and Schall, 2020; Schall, 2021). The life cycle is given. Because the form of the oocyst has been suggested as the single synapomorphy that will diagnose the monocystids, oocysts from parasites found in local earthworms were compared. Last, we provide gene sequence data for the new species and *Stomatocystis goerresi*, one of the parasites with a complex apical structure, and place the new species into a phylogeny with *M. agilis*, *Monocystis perplexa*, and *S. goerresi* and gregarines from other declared families to test the monophyly of the Monocystidae.

MATERIALS AND METHODS

Amynthas agrestis earthworms are epigeic, being found in the upper 5–10 cm of soil and litter, often with another invasive species, *Amynthas tokioensis*. Earthworms were collected at the type and 4 additional localities by manually sifting through soil from June to August 2021. The type host is either entirely parthenogenetic or sometimes sexual (Nouri-Aiin et al., 2022) and exhibits significant clonal variation among sites. All the adult *Amynthas* were identified using the morphological characters presented by Chang et al. (2016).

Earthworms were washed in dH_2O, killed in 50% ethanol, and washed again. After dissection, the body cavity was examined for the distribution of parasites. When located, parasites were removed to microscope slides in drops of earthworm saline solution (0.60 g NaCl, 0.012 g KCl, 0.02 g $CaCl_2$ in 100 ml dH_2O), a cover glass was applied, and the preparation was examined under a light microscope. Tissue in the preparation kept the cover glass from crushing the parasites. Measurements and photographs of each life stage were taken using the Moticam 1000 1.3MP Live Resolution microscope camera and Motic Image Plus 2.0.11 computer program (Motic, Richmond, British Columbia, Canada). Parasites of each life stage were measured using the guidelines of Clopton (2004). To count the number of oocysts per gametocyst, single gametocysts ($n = 3$) were removed to a fingerbowl and examined under ×30 magnification to determine if they were intact. Gametocysts were chosen from the middle range of diameters (700–800 μm). Each gametocyst was then ruptured in a known volume of earthworm saline solution (100 or 200 μl) and vortexed at 4,800 oscillations/minute, and then 0.5 or 1.0 μl

promptly placed on a microscope slide with cover glass, and every field was photographed. Oocysts were then counted and totaled for all photographs based on solution and sample volume. The gametocysts were fragile, so measurements of their diameter were taken in the dissected earthworm with a hand-held microscale (Minitool, Campbell, California). Counts of in situ gametocysts were estimated as <20, 20–100, 100–200, and >200.

Gametocysts from the new parasite were harvested by dissection and then burst to release the oocysts. These were washed several times in earthworm saline, then removed to a 2 ml thick-walled vial. *Stomatocystis goerresi* gametocysts were obtained by dissection of its host species, *Am. tokioensis* earthworms. These were also washed and removed to a 2 ml vial. The gametocysts of *S. goerresi* were opened by beating with a few 0.5 mm zirconia beads at 4,800 oscillations/minute (Mini Beadbeater, Biospec, Bartlesville, Oklahoma). Oocysts of each parasite species were then cleaned with 10% stock 7.5% sodium hypochlorite solution (7.1% available chlorine; Clorox Company, Oakland, California) for 10 min and washed with earthworm saline, and the oocysts opened by beating with a one-half volume of 0.5 mm zirconia beads at 4,800 oscillations/minute in lysis buffer from the DNeasy blood and tissue kit (Qiagen, Valencia, California), and the DNA extracted using the manufacturer's protocol.

The *18S SSU* rRNA gene was sequenced to recover a gene tree phylogeny for the parasites. This gene has been useful in such analysis for the Apicomplexa (Cavalier-Smith, 2014; Simdyanov et al., 2017), in part because it has both conserved and variable regions (Renoux et al., 2017). Therefore, the *18S SSU* rRNA gene was amplified in a single fragment (amplicon ~ 1,700 bases) using primers universal for eukaryotes, 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′ (Leander et al., 2003a). PCR amplification was done using the TopTaq kit (Qiagen) and the following PCR cycling protocol: 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. Amplicons were Sanger sequenced with both forward and reverse primer at the University of Vermont Genetic Analysis Core Facility.

A gene tree was constructed for the *18S* rRNA gene. Sequences for 18 gregarine species of 7 families including the new species, a new sequence for *Stomatocysis goerresi* (GenBank OP626898), and 16 others obtained from GenBank were aligned using MAFFT (Kazutaka et al., 2019) in Geneious Prime (Biomatters, Auckland, New Zealand) and used to build a consensus phylogeny using Jukes-Cantor neighbor-joining in Geneious Prime with 100 bootstrap replicates and *Loxomorpha harmothoe* as the outgroup. A Baysian analysis implemented in MrBayes (Ronquist and Huelsenbeck, 2003) (also within Geneious Prime) with *L. harmothoe* as the outgroup, produced the same tree topology and support (posterior probabilities for nodes), so only the neighbor-joining results are shown. The purpose of this analysis was not to create a phylogeny for the gregarines but to determine if the genera now placed into the Monocystidae are a monophyletic group.

To determine if any other earthworms in the area were hosts for the new parasite, other species were sampled from nearby areas in Chittenden County, Vermont. This was important to determine if the new parasite could have been transferred from another earthworm species. The species sampled were *Am. tokioensis* (n = 245), *Aporrectodea turgida* (n = 3), *Metaphire hilgendorfi* (n = 31), *Lumbricus terrestris* (n = 138), *Lumbricus rubellus* (n = 5), *Lumbricus festivus* (n = 1), *Octolaseon cyaneum* (n = 17), *Eisenia fetida* (n = 10), and *Dendrobaena octaedra* (n = 4).

As noted in the Introduction, a character that may be diagnostic for Monocystidae is the morphology of the oocyst. Therefore, oocysts for a variety of parasites were sampled, measured, and photographed from *Am. agrestis*, *Am. tokioensis*, and *D. octaedra* earthworms for comparison.

DESCRIPTION

Apolocystis bosanqueti n. sp.

(Fig. 1)

Phylum Apicomplexa Levine, 1988
Class Gregarinomorphea Grassé, 1953, emended by Cavalier-Smith (2014)
Order Eugregarinorida Léger, 1900, emended by Adl et al. (2019)
Family Monocystidae Bütschli, 1882
Genus *Apolocystis* Cognetti de Martiis, 1923

Description: Based on parasites from 23 *Am. agrestis* earthworms examined from the type locality with live wet mounts under cover glass using life stages defined by Levine (1971) and Clopton (2009) and shapes of the cells based on Clopton (2004).

Measurements given in micrometers as median (range), and (n = sample size). Parasites very rarely first seen as solitary gamonts, but most often as gamonts in association (thus precocious association). Gamonts (Fig. 1A–C) orbicular and the pair over time become a single orbicular form (Fig. 1D, E). Cells now gametocytes in this later association (n = 42) diameter 215.5 (146–338). Orbicular nucleus in each cell initially central, but often at the same edge as the gametocyst develops (Fig. 1E). Nucleus diameter (Fig. 1C) (n = 50) 40.0 (25–57). During syzygy paired gametocytes fuse closely and become gradually opaque (Fig. 1H). Zygotes appear massed within the now-opaque gametocyst (Fig. 1F, G) (n = 28) again orbicular, diameter 5.9 (5.2–6.7). Mature gametocysts white orbicular (Fig. 1J, K), readily seen through host body wall and often in large masses of hundreds and ranging in size from ~100 to ~1,000. Gametocyst epicyst gelatinous (Fig. 1I) (n = 8), 41.9 thick (35.5–66.5), and oocyst dehiscence occurs easily by simple rupture. Oocysts (Fig. 1L) (n = 30) fusiform with extended terminal tips (or hesperidiform) length 10.8 (9.8–11.9) and width 5.6 (5.1–6.3). Number of oocysts within 3 gametocysts counted = 110,800, 110,889, 116,550.

Taxonomic summary

Type host: Amynthas agrestis Goto & Hatai Megascolecidae.

Other hosts: The only other earthworm species found with similar morphology of gametocysts was *Am. tokioensis* (Beddard) Megascolecidae.

Type locality: University of Vermont Centennial Woods, Burlington, Vermont, 44°28′32.5″N, 73°11′13.5″W.

Other localities: University of Vermont Horticulture Research and Education Center (HREC), South Burlington, Vermont, 44°25′53.4″N, 73°11′57.2″W; Colchester Civic Center (CCC), Colchester, Vermont, 44°32′11″N, 73°12′19″W; Montpelier, Vermont, private home (M), 44°16′7.1″N, 72°33′58.1″W; Cady's Falls, Morrisville, Vermont, public garden (CF), 44°34′20.1″N,

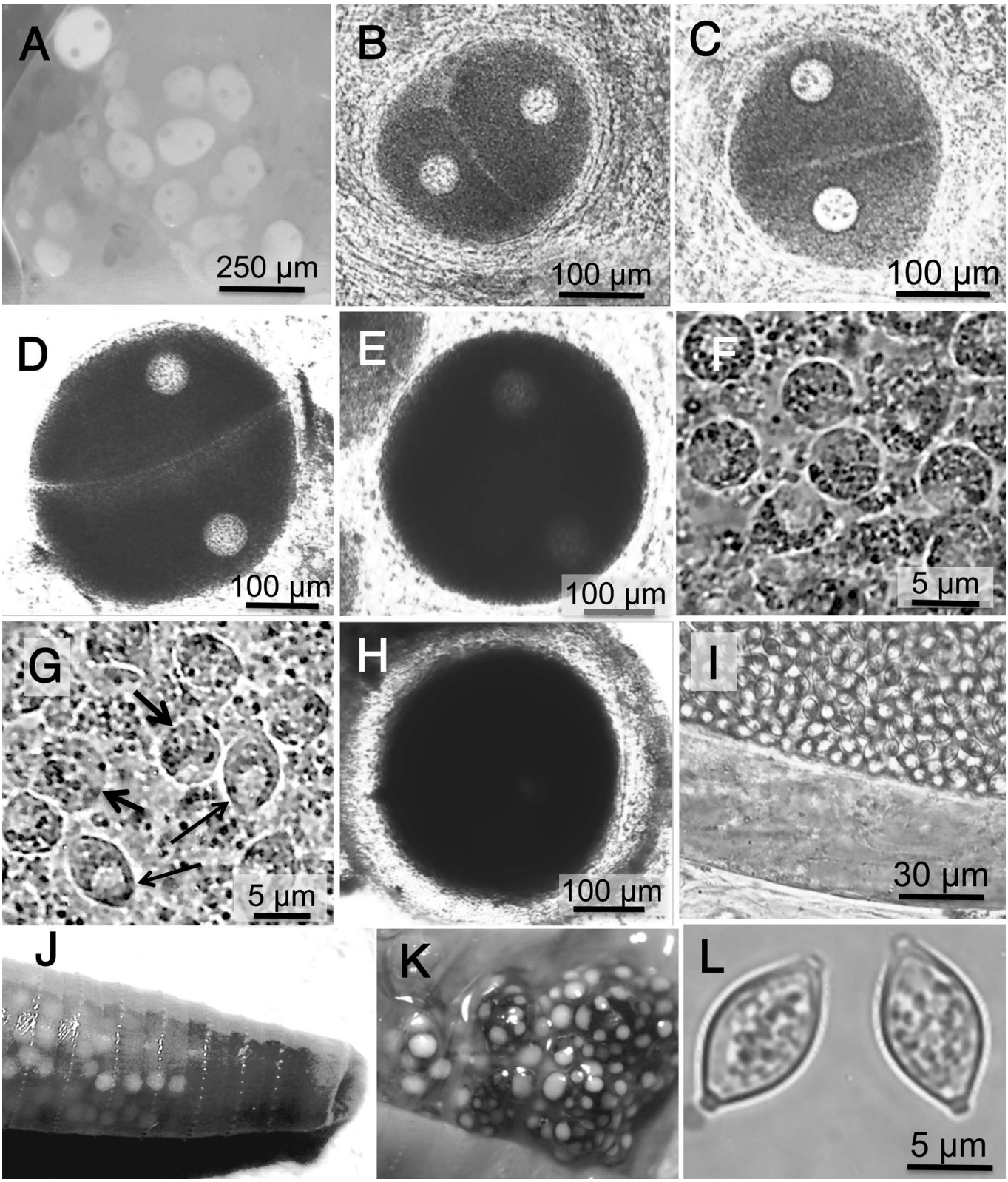

Figure 1. Life stages of *Apolocystis bosanqueti* n. sp. in the earthworm *Amynthas agrestis*. (**A**) Pairs of gamonts in association within the host tissue; nuclei are visible. (**B**) Pair of gamonts within host tissue in close association; nuclei visible. (**C**) Later paired gamonts now within an early gametocyst. (**D**) Paired gamonts in early opaque gametocyst. (**E**) Gametocytes in syzygy, very opaque, and nuclei very faint. (**F**) Zygotes within a mature gametocyst. (**G**) Zygotes developing into early oocysts; heavy arrows to zygotes and light arrows to early oocyst. (**H**) Mature gametocyst, completely opaque, showing thick envelope (epicyst). (**I**) View of gametocyst, now full of oocysts, and thick epicist. (**J**) Anterior segments of host earthworm with mature gametocysts visible though body wall, with largest gametocyst ~1 mm in diameter. (**K**) Dissected anterior of earthworm showing gametocysts as white spheres, largest ~1 mm. (**L**) Mature oocysts.

72°37′22.1″W. Maximum distance between sites 57 km (CCC to M).

Site of infection: Attached to anterior digestive tract, from esophagus, and posterior to the gizzard (segments 5–15).

Mode of transmission: Late in the season, gametocysts become fragile, the anterior end of the host earthworm filled with red fluid, and easily bursts when handled, all suggesting deposition of oocysts into the soil when the earthworm dies.

Prevalence and intensity of infection: During later summer (July–August) in *Am. agrestis* at type locality 149/166 (90%). At other localities: HREC 9/38 (24%), CCC 6/15 (40%), M 2/26 (8%), CF 6/11 (55%). Estimated number of gametocysts in a sample of *Am. agrestis* from type locality <20 (n = 28), 20–100 (n = 92), 100–200 (n = 17), >200 (n = 10).

Specimens deposited: Holotype and paratypes deposited at United States National Museum (1675956 [Holotype], 1675957, 1675958, 1675959).

ZooBank registration: urn:lsid:zoobank.org:act:663BDABF-6DF0-4435-8D78-A3C732DB8F57.

Etymology: Species epithet named for W. Cecil Bosanquet (1866–1941), the distinguished medical researcher and educator who first described a parasite species related to *Ap. bosanqueti*. Pronunciation guide for species epithet: "bōz-an-ket-i."

Molecular sequences/DNA sequences: 18S small subunit ribosomal RNA gene (1,677 bp, GenBank OP626897). Sequences for *Ap. bosanqueti* recovered from *Am. agrestis* earthworms from type locality CW (n = 3), HF (n = 3), and CF (n = 1) were identical.

Remarks

Bosanquet (1894) described *Apolocystis* (*Monocystis*) *herculea* as a gregarine parasite of the British earthworm, *Lumbricus herculeus*. The parasite was distinctive in producing large gametocysts visible through the earthworm's body wall as white spheres congregated in the posterior body cavity. The species' epithet referenced both the host and the large size of the gametocysts, which were visible to the naked eye. Since then, parasites with this striking gametocyst stage have been reported from England, Wales, Sweden, France, Germany, Poland, Bulgaria, and the former Czechoslovakia and have all been considered as *A. herculea* (reviews in Pizl, 1990 and Segun, 1968). The reported host range is broad, fully 15 species across 8 genera. All but 1 of these is in the large earthworm family Lumbricidae. This large host and geographic distribution likely masks undescribed species of parasite. Indeed, Rees (1963) noted variations in the parasite size and life history in different earthworm host species.

Apolocystis bosanqueti resembles *Ap. herculea* in producing large spherical gamonts without polar differentiation that develop into large white gametocysts in the earthworm's body cavity but is distinguished by its location in the anterior end of the host attached to the esophagus both anterior and just posterior to the crop/gizzard. Rees (1963) describes a life cycle for *Ap. herculea* that differs greatly from that of *Ap. bosanqueti*, and that author states that the parasite appears as large trophozoites in the body cavity that form white cysts yielding many daughter trophozoites that then join to produce the final gametocyst and oocysts. *Apolocystis bosanqueti* displays an apparent precocious association of gamonts, the pairs appearing in the anterior tissues of the earthworm, which form the gametocysts. Occasionally 2 or 3 pairs of gamonts are within the developing gametocyst, but these are likely to be a result of pairs of gamonts closely associated in the earthworm's tissue. The only other *Apolocystis* with large white gametocysts located in the anterior gut of the earthworm is *Apolocystis proventus* (Ramadan et al., 2015), also from a megascolecid host, but is markedly smaller in all stages than *A. bosenqueti* (maximum diameter of the *Ap. proventus* gametocyst is 88 μm vs. >1,000 μm for *Ap. bosenqueti*).

The type host of *Ap. bosanqueti* is *Am. agrestis*, family Megascolecidae, which originated in Japan and is an invasive species in North American forests (Keller et al., 2017; Nouri-Aiin et al., 2020). Thus, the host and geographic range of *Ap. bosanqueti* differ from other similar parasites.

We place the new species into *Apolocystis*, although this genus is problematic in lacking diagnostic characters. It is described as simply a spherical cell, with no real distinguishing morphological characters. Indeed, Segun (1968) presents a figure for *Apolocystis* as simply a circle. Kundu and Bandyopadhyay (2019) provide a list of 41 described *Apolocystis* species, with most from the earthworm seminal vesicles, and only 9 from the coelom. A distinction is the thickness of the gametocyst epicyst, being thin, tough hyaline for species in the seminal vesicle (similar to *Monocystis*; see Crespi et al., 1981), or thick gelatinous for those attached to the gut or body wall, such as *Ap. proventus* and *Ap. bosanqueti*. This may reflect a systematically important character.

DISCUSSION

The taxonomy of the gregarine family Monocystidae is confounded. Bütschli (1882) is given as the authority for the family in most reviews and species descriptions (for example, Levine, 1988). However, no formal description of the family appears in Bütschli (1882). Instead he refers to the order Monocystidea of Stein (1848) and then discusses species with "Monocystid" characters following descriptions given by previous authors (Kölliker, 1848; Stein, 1848). The term monocystid in those publications simply means "single bladder" to distinguish, in modern terms, the aseptate from the septate gregarines. Thus, the Monocystidae would include any aseptate gregarine. Later, Monocystidae became widely accepted as a taxon to include aseptate gregarines with a spherical to cylindrical trophozoite, no or slight differentiated pole(s), and the distinctive morphology of oocysts. Although Bhatia (1930) made a reasonable case to divide the family based on the morphology of the trophozoites into several new families, the morphological form of the oocysts is noted to unite the families into a taxonomic tribe.

To test the monophyly of the monocystids, we recovered a single-gene phylogeny that included 4 species of 3 genera now placed into the Monocystidae as well as other gregarines selected from several morphologically distinct families (Fig. 2). Both topology and nodal support values for the neighbor-joining and Baysian analysis concord, which indicates support for the hypothesis of monophyly. In the tree, the monocystids fall into a well-supported clade, but relationships within the family are not resolved with no strong support for any node. The closest sister in the tree is *Prismatospora*, which agrees with previously published phylogenies that included a large sample of gregarine taxa, but only a single monocystid (Cavalier-Smith, 2014; Simdyanov et al.,

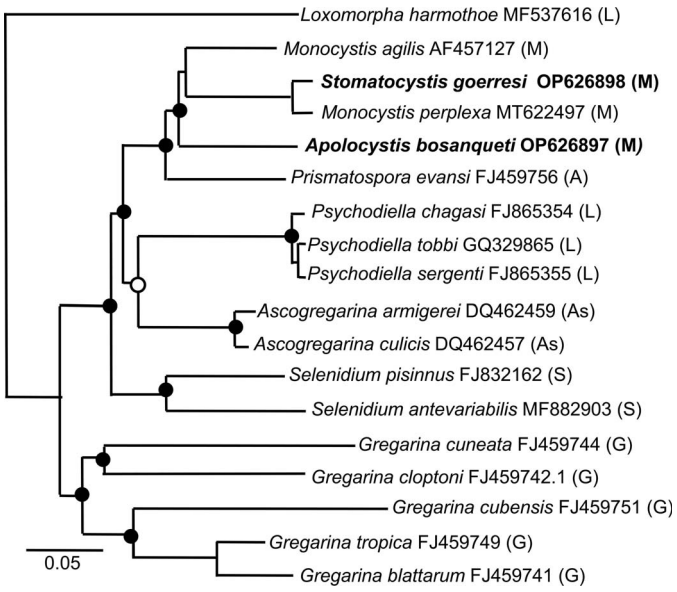

Figure 2. Phylogenetic tree for gregarine species from several families for 18 taxa showing species names and GenBank accession numbers including 2 new monocystid sequences shown in bold (*Apolocystis bosanqueti* n. sp. and *Stomatocystis goerresi*). Family codes are L = Lecudinidae; M = Monocystidae; A = Actinocephalidae; As = Ascogregarinidae; S = Selenidiidae; G = Gregarinidae. Shown is a Neighbor-Joining tree with genetic-distance bar (very similar topology for a Bayesian tree). Basal nodes with strong support (>0.99) by both Bayesian (posterior probabilities) and neighbor-joining methods (bootstrap values) are indicated with dots, and one node with high support only by Bayesian analysis shown with an open point.

2017). *Psychodiella* and *Ascogregarina* appear as sisters, as proposed by Lantova and Volf (2014). Species of *Gregarina* also appear in the tree in a well-supported clade, but in 2 distinct groups as proposed by Clopton (2009). Thus, the topology of the tree concords with previous studies that support the grouping of the monocystid parasites shown in Figure 2.

The single morphological trait that may diagnose the Monocystidae is the structure of the oocyst. Figure 3 shows a variety of oocysts, all photographed and shown at the same scale. Included are 3 genera of monocystids in the Figure 2 gene tree plus other oocysts sampled from earthworms at our study sites. Although the oocysts differ significantly in size, all have a similar morphology. Early workers called this form navicular ("navicella" or boat shape) such as Henle (1845), with recent authors using the terms biconical (Levine, 1977, 1988), biconical with bipolar plugs (Desportes and Schrével, 2013), and fusiform with distinctive apical tips (Keller and Schall, 2020). A similar form in other gregarines has been called hesperidiform (e.g., Clopton et al., 2008), meaning lemon-shaped, albeit elongated. The oocyst apical tips can be pronounced (Fig. 3A) or slight (Fig. 3B, G). The morphology of gamonts, the form of association of gamonts, and the sexual stages differ significantly for the 3 genera of monocystids in the figure. *Monocystis perplexa* has laterally fused gamonts in association and syzygy typical for the genus and production of isogametes around the perimeter (Keller and Schall, 2020). Gamonts of *Stomatocystis goerresi* join by the anterior funnel structures and then fuse to form a sphere where isogametes are produced in germinal centers (Schall, 2021). *Apolocystis bosanqueti* gamonts are spherical and merge to produce a spherical structure. The number of oocysts produced per gametocyst ranges from ~100 for *M. perplexa*, ~2,000 for *S. goerresi*, to ~100,000 for *Ap. bosanqueti*. The evidence for the monophyly of the monocystids includes both the molecular results and oocyst morphology. Only a single study on the ultrastructure of an *Apolocystis* oocyst has been reported (Crespi et al., 1981), so the homology of the oocyst morphology seen in Fig. 3 is not resolved.

Earthworms have a worldwide distribution, and because they are not tolerant of submersion in salt water, that distribution must reflect continental drift over 100 million years (Phillips et al., 2019). Monocystids are found in earthworms worldwide (Desportes and Schrével, 2013; Keller and Schall, 2020), so they must also be an ancient group. Over that time the parasites have thus evolved great morphological and life cycle variation, but the sole morphological trait that may be a synapomorphy is the oocyst form. The conservative nature of the oocyst morphology could be functional, perhaps for optimal packing into the spherical gametocyst. However, estimating the volume of an *Ap. bosanqueti* oocyst using an ellipsoid form and the volume of a gametocyst (minus the thick epicyst) reveals that there would be room for ~5 times the number of oocysts that were counted. Borengasser and Clopton (2019) also noted the loose packing of cylindrical oocysts of 3 species of gregarines within the mature gametocyst. Thus, the packing of oocysts into the gametocyst may not be the driver of the conserved morphology of monocystid oocysts. The form may allow optimal packing of the elongated 8 sporocysts within the oocyst or aid in survival in the soil. The oocysts must remain in the soil from November to April when the earthworms exist only as eggs within a tough cocoon, and we have noted that during drought years the earthworms are almost absent, to return in large numbers during a rainy year. The parasite oocysts must therefore remain viable in the soils over long periods, even years, which is known for other gregarines (Clopton et al., 2016).

The type locality for *Ap. bosanqueti* is a site in northern Vermont, but we propose that the source for this parasite is likely Japan, and thus the range of *Apolocystis* is extended to East Asia, albeit indirectly in an invasive host now in North America.

This conclusion must be defended because the parasite may be present in naturalized earthworms originating from Europe, where *A. herculean* is widespread and has crossed into the more recent invasive earthworms from Japan. The type and the additional host of *Ap. bosanqueti* are *Am. agrestis* and *Am. tokiensis* earthworms that were introduced into North America from Japan (Nouri-Aiin et al., 2020). Parasites with large spherical white gametocysts may be present in other Japanese earthworms. A guide to the earthworms of Japan presents a photograph of another megascolecid, *Pheretima conformis*, with clear gametocysts seen through the body wall, but no mention is made in the text (Ishizuka and Minagoshi, 2014). Worthy of note is that despite many species of earthworms in Europe that are hosts to parasites described as *A. herculea*, none of 6 introduced earthworm species from Europe now naturalized in Vermont that were sampled (n = 178) were found infected with a similar parasite. Last, all the *18S SSU* rRNA gene sequences from *Ap. bosanqueti* from 3 sites were identical, which is expected if the earthworms were introduced from Japan recently.

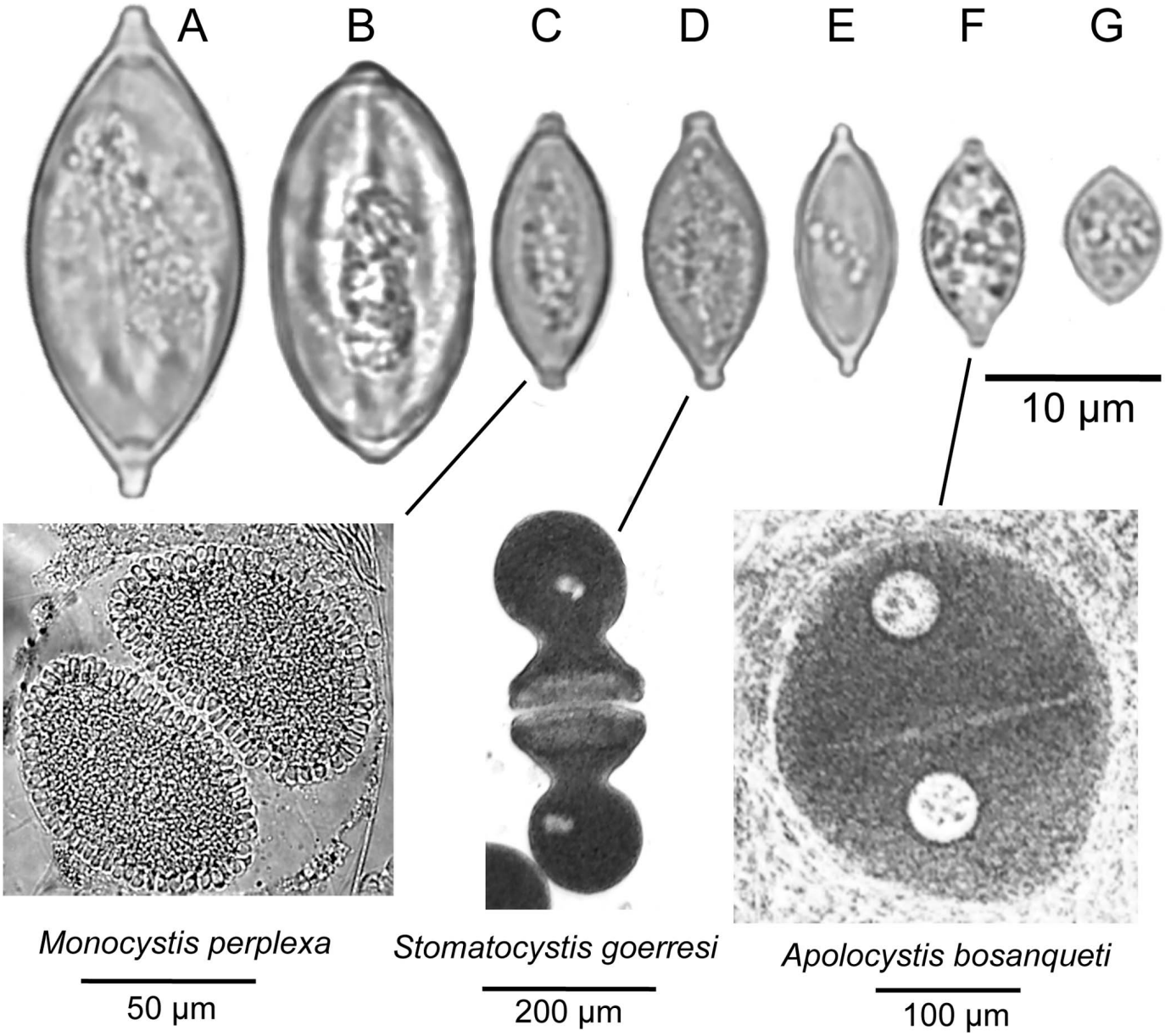

Figure 3. Oocysts (shown at same scale) from 3 known monocystids, also showing their gamonts in association (*Stomatocystis*, and *Apolocystis*) or gametocytes in syzygy (*Monocystis*). Also shown are oocysts from 4 other species of likely monocystids recovered from earthworms at the study site. (**A**) Oocyst from introduced European earthworm *Dendrobaena octaedra*. (**B**) Oocyst from earthworm *Amynthas agrestis*. Gametocysts of this species produce 5–10 oocysts. (**C**) *Monocystis perplexa* from *Am. agrestis*. (**D**) *Stomatocystis goerresi* from *Amynthas tokioensis*. (**E**) Oocyst from *D. octaedra*. (**F**) *Apolocystis bosanqueti* from *Am. agrestis*. (**G**) Oocyst from a species that produces small (~100 µm diameter) gametocysts attached to the posterior gut of *Am. agrestis*.

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