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Cambaraspora faxoni n. sp. (Microsporidia: Glugeida) from native and invasive crayfish in the USA and a novel host of *Cambaraspora floridanus*

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ABSTRACT

Crayfishes are among the most widely introduced freshwater taxa and can have extensive ecological impacts. Knowledge of the parasites crayfish harbor is limited, yet co-invasion of parasites is a significant risk associated with invasions. In this study, we describe a novel microsporidium, *Cambaraspora faxoni* n. sp. (Glugeida: Tuzetiidae), from two crayfish hosts in the Midwest USA, *Faxonius virilis* and *Faxonius rusticus*. We also expand the known host range of *Cambaraspora floridanus* to include *Procambarus spiculifer*. *Cambaraspora faxoni* infects muscle and heart tissue of *F. rusticus* and develops within a sporophorous vesicle. The mature spore measures 3.22 ± 0.14 μm in length and 1.45 ± 0.13 μm in width, with 8–9 turns of the polar filament. SSU sequencing indicates the isolates from *F. virilis* and *F. rusticus* were identical (100%) and 93.49% similar to *C. floridanus*, supporting the erection of a new species within the *Cambaraspora* genus. The novel parasite was discovered within the native range of *F. rusticus* (Ohio, USA) and within a native congeneric (*F. virilis*) in the invasive range of *F. rusticus* (Wisconsin, USA). *Faxonius virilis* is invasive in other regions. This new parasite could have been introduced to Wisconsin with *F. rusticus* or it may be a generalist species with a broad distribution. In either case, this parasite infects two crayfish species that have been widely introduced to new drainages throughout North America and could have future effects on invasion dynamics or impacts.

1. Introduction

Microsporidia are a group of spore-forming, intracellular parasites that infect a high diversity of hosts (Murareanu et al. 2021). Microsporidian systematics have recently undergone an updated phylogenetic framework which emphasized genetic/genomic data with supporting data related to morphology, development, ultrastructure, and ecology (Bojko et al. 2022; Wijayawardene et al. 2022). This framework reintroduced a classical naming system for the Microsporidia, which removed clade-based taxonomic ranking (e.g. I, II, III, IV, V) and now includes seven taxonomic groups within the Rozellomycota and Microsporidia (Amblyosporida, Enterocytozoonida, Glugeida, Neopereziida, Nosematida, Caudosporida, and Ovavesiculida) (Bojko et al. 2022).

The Glugeida group primarily contains aquatic microsporidia from fish and crustacean hosts (Bojko et al. 2022). Members of this group have been found to have a broad host range or even infect multiple host groups (Stentiford et al. 2018; Bojko et al. 2020a; Stratton et al. 2022a; Stratton et al., 2023). The Glugeida group houses eight microsporidian families identified to date, including the Tuzetiidae (Larsson, 1983).

The Tuzetiidae family currently hosts 11 microsporidian species from five genera, including: *Nelliemelba*, *Pankivauam*, *Paratuzetia*, *Tuzetia*, and *Cambaraspora* (Bronnvall and Larsson, 1995; Cheney et al. 2000; Canning et al. 2002; Poddubnava et al. 2006; Simakova et al. 2009; Bojko et al. 2020b; Bojko et al. 2022; Wijayawardene et al. 2022). All members of this family infect aquatic invertebrate hosts, primarily freshwater ephemeropterans and crustaceans in Europe and Russia (Larsson 1983; Simakova et al. 2009). However, genetic data exists for only two species within the Tuzetiidae family, where the other species were placed based on morphological and ultrastructural data (Bronnvall and Larsson, 1995; Poddubnava et al. 2006). The first Tuzetiidae member with genetic data was *Tuzetia weidneri* infecting *Panaeus aztecus* (brown shrimp) from a bayou in Mississippi, USA (Cheney et al. 2000;

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Canning et al. 2002). More recently, *Cambaraspora floridanus* was described from *Procambarus fallax* (slough crayfish), with supporting genetic data (Bojko et al. 2020b). The *Cambaraspora* genus was originally included in the Glugeidae family; however, an updated phylogeny supports the species and genus as a part of the Tuzetiidae family (Bojko et al. 2020b; Bojko et al. 2022). In addition, the genus *Fusasporis* has not been formally placed into a family, but phylogenetic analysis may also support its placement at the base of the Tuzetiidae and Glugeidae families (Lovy et al. 2021; Bojko et al. 2022). Using genetic information, the Tuzetiidae appears to house only crustacean-infecting species from the USA.

The *Cambaraspora* genus contains a microsporidium, *Cambaraspora floridanus*, that infects crayfish hosts. This genus was introduced as a 'complex', whereby one species was adequately identified using morphological, pathological, developmental, and molecular evidence and other DNA sequence isolates were documented without ultrastructural examination (Bojko et al. 2020b). Based on this evidence, *Cambaraspora floridanus* infects *Procambarus fallax* and *Procambarus paeninsulanus* in Florida. The other DNA sequence isolates were documented from Florida dwarf crayfish species (*Cambarellus blacki* and *Cambarellus shufeldtii*) and were ~ 2% dissimilar (Bojko et al. 2020b). Each host exhibited a muscular infection with white muscle tissue in heavily infected areas. The parasite developed via a sporophorous vesicle, which included 32 spores per vesicle. The final spore was elongate with 19–21 turns of the polar filament. The parasite was only identified in Florida, leading to the species *C. floridanus*; however, broader surveillance was not possible to determine whether isolates of this genus were present outside of Florida.

We provide evidence for a novel crayfish-infecting microsporidium within the genus *Cambaraspora* (Glugeida: Tuzetiidae), while also expanding the known host range of *C. floridanus* by screening additional Florida crayfish. We provide histological, genetic, phylogenetic, and ultrastructural data to describe *Cambaraspora faxoni* n. sp., which infects *Faxonius rusticus* and *Faxonius virilis* from Midwest USA freshwaters.

2. Material and methods

2.1. Crayfish locality and collection

Fifty-five *F. rusticus* were collected from their native range in Ohio, USA on the 30th of June 2021 and checked for signs of gross pathology in the form of white musculature. A single *F. rusticus* female with white musculature was collected from this site (Table 1). The individual was shipped overnight to the University of Florida Fisheries and Aquatic Sciences laboratory where it was dissected for histopathology. In addition, 10*F. virilis* were collected from their native range in Wisconsin, USA (invasive range of *F. rusticus*) on the 15th of July 2020 and checked for signs of gross pathology. A single *F. virilis* female with white musculature was collected (Table 1). A piece of abdominal muscle tissue from this individual was fixed in 96% molecular grade ethanol. Finally, a female *Procambarus spiculifer* was collected from its invasive range in Florida, USA on the 12th of September 2021 (Table 1). This individual did not present with signs of gross pathology at the time of capture but later died in the laboratory. Abdominal muscle tissue from the individual was fixed in 96% molecular grade ethanol.

2.2. Histopathology

The *F. rusticus* individual was dissected for histopathological screening, which included fixing the following tissues in Davison's Freshwater Fixative (DFWF; tap water, glacial acetic acid, ethanol, and formaldehyde): nerve tissue, uropod, muscle, gut, hepatopancreas, heart, gonad, gill, eye, and antennal gland. After 24 h, the preserved tissues were moved to 70% ethanol. The sample was histologically processed by Histology Tech Services (Gainesville, FL, USA) where the tissues underwent a dehydration and xylene exchange followed by paraffin wax infiltration. The wax-infiltrated tissues were then embedded into wax-blocks, sectioned (3–4 μm), and mounted onto a glass slide. Mounted sections were stained using haematoxylin and alcoholic eosin after being dewaxed and rehydrated. Histological screening was conducted using a Leica DM500 light microscope and images were acquired using an integrated Leica camera system.

2.3. Transmission electron microscopy

A biopsy of muscle tissue was collected from *F. rusticus* during dissection and preserved in 2.5% glutaraldehyde in 0.1% sodium cacodylate buffer for transmission electron microscopy (TEM). The sample was transferred to 4% paraformaldehyde with 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.24). Sample processing was completed with the assistance of a Pelco BioWave Pro laboratory microwave (Ted Pella, Redding, CA, USA). The sample was secondarily fixed in 2% osmium tetroxide after a 0.1 M sodium cacodylate (pH 7.24) wash. The sample was water washed and dehydrated in a graded ethanol series (25% to 100% in 5% increments followed by 100% acetone). The dehydrated sample was infiltrated in increments 3:1, 1:1, and 1:3 of anhydrous acetone:ARALDITE/Embed epoxy resin and Z6040 embedding primer (Electron Microscopy Services (EMS), Hatfield, PA, USA) followed by 100% ARALDITE/Embed. Semi-thin sections (500 nm) stained with toluidine blue were collected from resin infiltrated samples after curing for 72 h at 60 °C. Ultra-thin sections were stained with 2% aqueous uranyl acetate and citrate (EMS, Hatfield, PA, USA) after being collected on carbon coated Formvar 100 mesh grids (EMS, Hatfield, PA, USA). An FEI Teenai G2 Spirit Twin TEM (FEI Corp., Hillsboro, OR, USA) was used to examine sections. Digital images were acquired using a Gatan UltraScan 2 k × 2 k camera and Digital Micrograph software (Gatan Inc., Pleasanton, CA, USA). ImageJ software was used to measure mature spores imaged from TEM (Schneider et al. 2012).

2.4. Molecular diagnostics

Microsporidian-infected muscle tissue from all three individuals underwent a DNA extraction using a Qiagen DNEasy Extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Extracted DNA was used in a Promega 'Flexi-Taq' PCR (Promega, Madison, WI, USA) consisting of 10 μL buffer, 2.5 mM $MgCl_2$, 1 mM dNTPs, 1 μM forward primer V1F (5′-CACCAGGTTGATTCTGCCTGAC −3′), 1 μM reverse primer MC3r (5′-GATAACGACGGGCGGTGTGTACAA

Table 1
Detailed information on each individual crayfish's locality, sex, carapace length, and the associated microsporidian data.

Host Species	Site	Range	Coordinates	Collection Date	Sex	Carapace Length (mm)	Microsporidian Species	SSU	Histology	TEM	Accession Number
Faxonius rusticus	Little Darby Creek, OH	Native	40.01338, −83.38318	30 June 2021	F	35	*C. faxoni* n. sp.	✓	✓	✓	OQ672255
Faxonius virilis	South Fork Main Creek, WI	Native	45.53711, −90.71047	15 July 2020	F	21	*C. faxoni* n. sp.	✓	—	—	OQ672256
Procambarus spiculifer	Camp Blanding, FL	Invaded	29.94151, −81.95660	12 September 2021	F	36	*C. floridanus*	✓	—	—	OQ672257

−3′), and 0.25 μL Promega Taq polymerase in a 50 μL reaction (Ovcharenko et al. 2010). The thermocycler conditions included an initial denaturization at 94 °C for five minutes followed by 35 cycles of 94 °C–55 °C–72 °C, with each temperature held for one minute. Followed by a final extension period for seven minutes at 72 °C. The resulting amplicons (~1100 bp) were visualized on a 1.5% agarose gel using gel electrophoresis. The microsporidian bands were excised from the gel and extracted using a Qiagen Gel Extraction kit (Qiagen, Hilden, Germany). The extracted samples were sent for Sanger sequencing using Eurofins Genomics (www.eurofinsgenomics.com).

2.5. Phylogenetics and genetic comparisons

A maximum-likelihood (ML) phylogenetic tree was constructed to compare our novel isolates to 120 microsporidian SSU sequences representing each major microsporidian group with a *Metchnikovella* isolate as the outgroup. Sequences were MAFFT aligned using CIPRES resulting in 2483 comparative columns including gaps (https://www.phylo.org/). The alignment was analyzed using the IQtree web server (http://iqtree.cibiv.univie.ac.at/), which tested 88 different evolutionary models and resulted in the application of the GTR + F + I + G4 evolutionary model, which was selected as the best fitting model according to Bayesian information criterion (Trifinopoulos et al. 2016). The final tree was constructed from 1000 bp replicates. In addition, the Sequence Demarcation Tool (SDT v.1.2) was used to compare the genetic similarity between microsporidian species from the Tuzetiidae and Glugeidae.

3. Results

3.1. Gross pathology and histopathology

During collection, all individuals were checked for signs of gross pathology including 10*F. virilis* and 55*F. rusticus*. A single *F. rusticus* and a single *F. virilis* presented with signs of gross pathology in the form of white musculature visible beneath the cuticle of the abdomen upon collection (Fig. 1a). The *P. spiculifer* host did not present with signs of gross pathology. Histopathology of *F. rusticus* featured microsporidian spores developing within sporophorous vesicles (SPV) in the sarcolemma of host skeletal muscle fibers (Fig. 1b-d) and heart myocardium (Fig. 1e) with multiple developmental stages evident in both tissue types. An immune response was observed within the heart tissue resulting in the production of a granuloma (Fig. 1e).

3.2. Ultrastructure and intracellular microsporidian development cycle

TEM of infected muscle tissue collected from *F. rusticus* provided details of the novel microsporidium's developmental pathway and ultrastructure. The developmental pattern of our novel isolate followed a similar pattern to *C. floridanus*, but stark differences in the mature spore morphology were found (Table 2; Bojko et al. 2020b). Merogony and sporogony were observable throughout the muscle tissue and, aside from the meront, each spore stage developed within individual SPVs, with SPVs at varying developmental stages found in close proximity to one another (Fig. 2a). Development began with a putative unikaryotic meront in direct contact with the host cell cytoplasm (Fig. 2b). The nucleus of the unikaryotic meront divided to produce a diplokaryotic meront with a slightly thickened cell membrane (Fig. 2c). A sporogonial plasmodium was observed, which is assumed to divide into up to 32 unikaryotic sporonts, since up to 27 spores were observed within a single

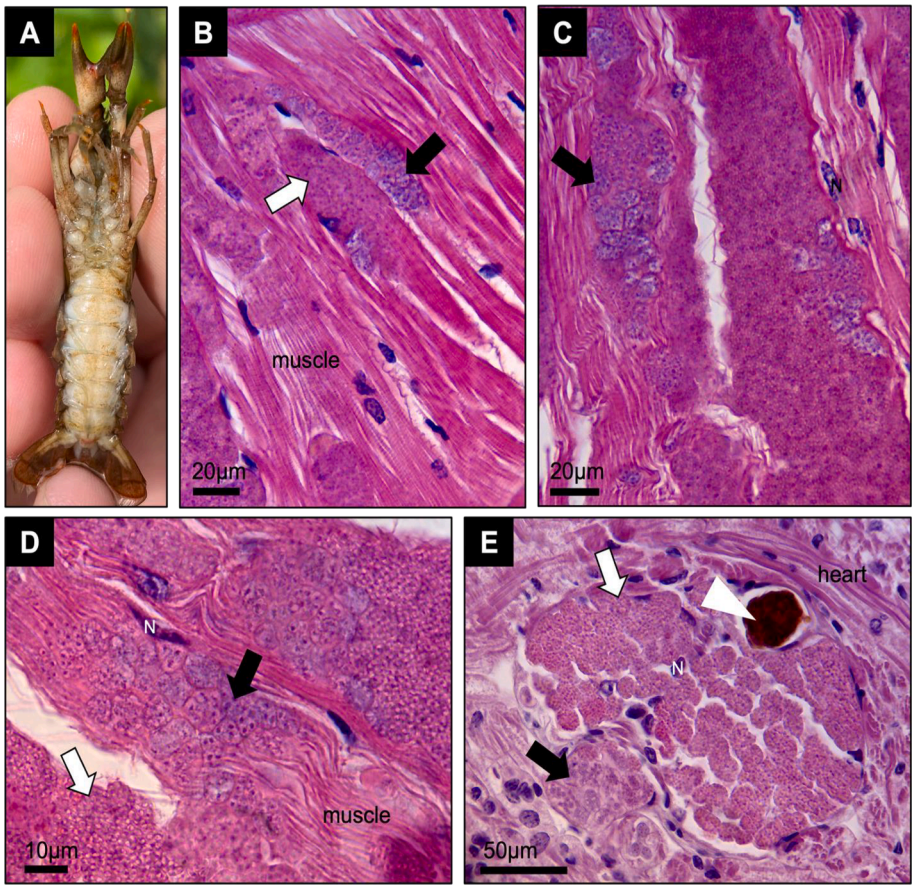

Fig. 1. Gross pathology and histopathology of *Cambaraspora faxoni* n. sp. (A) *Faxonius virilis* presenting with white musculature visible through the ventral cuticle of the abdomen. (B) Muscle tissue of *Faxonius rusticus* infected with microsporidian parasite developing within sporophorous vesicles (SPV). Early (black arrow) and late (white arrow) stages of development were observed. (C) Additional early sporogony (black arrow) observed near a heavily infected area where muscle tissue is beginning to rupture. (D) High-magnification image (40X) of early (black arrow) sporogony developing in close proximity to a host nucleus of muscle tissue. Mature spores within SPVs observed (black arrow). (E) Microsporidian infection observed within myocardium of heart resulting in the development of a granuloma (white arrowhead). Packets of mature spores (white arrow) and early sporogony (black arrow) were present.

Table 2
Morphological comparison of the two described *Cambaraspora* species.

Morphological Feature	*C. faxoni* n. sp.		*C. floridanus*[1]	
Spore shape	Oval, wider posterior end		Cucumiform	
Spore length (μm)	3.22 ± 0.14	n = 6	6.14 ± 0.84	n = 12
Spore width (μm)	1.45 ± 0.13	n = 10	2.12 ± 0.23	n = 12
Spore wall (nm)	83.10 ± 15.01	n = 10	150.83 ± 22.69	n = 10
Spore wall at apex of spore (nm)	55.22 ± 10.63	n = 9	91.09 ± 23.19	n = 10
No. coils in polar filament	8–9		19–21	

[1] Bojko et al. 2020b.

SPV (Fig. 2d-e). Early unikaryotic sporonts developed a thick electron-dense plasmalemma as they progressed to sporoblasts with early signs of electron-dense organelles (Fig. 2f-g).

During sporogony the plasmalemma continued to thicken and organelles proceeded to mature (Fig. 3a). As spores developed towards maturity, they developed a spore wall composed of an electron-lucent endospore and electron-dense exospore (Fig. 3b). The mature spores were diplokaryotic (Fig. 3c), suggesting that an unnoticed nuclear division takes place, and developed an anchoring disk, bi-layered polaroplast, and an anisofilar polar filament (Fig. 3d). The mature spores were oval-pear in shape with a wider posterior end measuring 3.22 ± 0.14 μm (n = 12, SD) in length and 1.45 ± 0.13 μm (n = 12, SD) in width. The spore wall was 83.10 ± 15.00 nm (n = 10, SD) thick, except where it thinned at the apex of the spore above the anchoring disk, where it measured 55.22 ± 10.63 nm (n = 10, SD) (Fig. 3e). The polar filament coiled 8–9 times around the periphery of the cell (Fig. 3f). Morphological plasticity of the maturing spore was observed within some SPVs, where some spores were noted to develop much larger than their sister spores within the same SPV (Fig. 3g).

3.3. Genetic similarity and phylogenetic placement of novel isolates

The microsporidian SSU sequence from *F. rusticus* (OQ672255: 1017 bp) and *F. virilis* (OQ672256: 1008 bp) were 100% identical. The 1017 bp sequence from *F. rusticus* was most similar to the *C. floridanus* isolate from *Cambarellus shufeldtii* (MT006313: 100% coverage; 93.49% sim.; e-value: 0.0). The microsporidian SSU sequence from *P. spiculifer* (OQ672257: 961 bp) was most similar to the *C. floridanus* isolate from *Procambarus fallax* (MT006316: 96% coverage; 99.68% sim.; e-value: 0.0).

The ML phylogeny placed the new isolates within the Glugeida (100% bootstrap support) and within the Tuzetiidae (85% bootstrap support) (Fig. 4). The new isolates infecting *F. rusticus* and *F. virilis* branched outside of the existing *Cambaraspora* group as a novel branch that was associated with the genus *Cambaraspora* (Glugeida: Tuzetiidae) (83% bootstrap support). Branching below it with 85% confidence was *Tuzetia weidneri* (AJ252958: 87.43% similarity; 74% coverage; e-value: 0.0) isolated from *Penaeus* (=*Farfantepenaeus*) *aztecus* in Mississippi. The isolate infecting *P. spiculifer* branched within the *C. floridanus* species complex, with 100% supported association with the *P. paeninsulanus* and *P. fallax* isolates.

A sequence demarcation plot including all known *Cambaraspora*, *Tuzetia*, *Glugea,* and *Fusasporis* isolates highlighted that *Glugea* remained the most similar microsporidian genus to the *Cambaraspora*, despite the phylogenetic topology clustering the isolates more closely with *Tuzetia* (Fig. 4).

4. Taxonomic summary

4.1. Higher taxonomy

Superphylum: Opisthosporidia Karpov et al. (2014).

Phylum: Rozellomycota (Tedersoo et al. 2018), including Microsporidia (Balbiani, 1882; Wijayawardene et al. 2020).

Class: Marinosporidia Vossbrinck et al. (2014).

Order: Glugeida Bojko et al. (2022).

Family: Tuzetiidae Larsson (1983).

Genus: *Cambaraspora* Bojko et al. (2020b).

Genus description: *"Members of this genus infect the muscle tissue of aquatic crustacean hosts and undergo merogony and sporogony in a sporophorous vesicle. The spores are diplokaryotic and include between 8 and 21 coils of the polar filament. The polaroplast is bi-laminar, with an electron dense upper layer and electron lucent lower layer. The spores are cucumiform to oval or pear-shape. Multiple developing life stages are evident in a single sporophorous vesicle (<32). Genetic relatedness to the type species should be considered if placing a novel member into this genus"* – amend. Bojko et al. 2020b.

4.2. Species description

Novel species: *Cambaraspora faxoni* n. sp. Stratton, Kabalan, Bolds, Reisinger, Behringer, and Bojko, 2023.

Species description: This species infects the muscle tissue of *Faxonius* spp., undergoing merogony and sporogony within a sporophorous vesicle. The mature spores are oval-pear in shape with a wider posterior end measuring 3.22 ± 0.14 μm (SD) in length and 1.45 ± 0.13 μm (SD) in width. Mature spores are diplokaryotic with 8–9 turns of the polar filament. To belong within this species, the small sub-unit ribosomal RNA gene must be within 98% similarity to that sequenced herein.

Type host: *Faxonius rusticus* (Decapoda: Cambaridae).

Type locality: Little Darby Creek (40.013388, −83.38318), Madison County, Ohio, USA.

Site of infection: This species infects the muscle and heart tissue of the host.

Etymology: The species *Cambaraspora faxoni* is named for the host genus (*Faxonius*).

Type material: Resin blocks, histology slides, glutaraldehyde-fixed tissue, and ethanol-fixed tissue are stored at the University of Florida, Reisinger Laboratory. Genetic data are reposited in NCBI under accession: OQ672255.

5. Discussion

Crayfish are an important group of ecologically relevant crustaceans. They are important in freshwater ecosystems as omnivores that often impact several trophic levels and are a critical resource for many higher order predators (Wilson et al. 2004; DiStefano, 2005). This group of crustaceans can be invasive, and they can carry invasive parasites (Bojko et al. 2021). Globally, crayfish have been found with microsporidian parasites from ten species, within five genera: *Alternosema*, *Astathelohania* (previously *Thelohania*), *Cambaraspora*, *Nosema* (previously *Vairimorpha*), and *Ovipleistophora* (Moodie et al., 2003a; Moodie et al., 2003b; Moodie et al., 2003c; Pretto et al., 2018; Bojko et al., 2020a; Bojko et al., 2020b; Tokarev et al. 2020; Stratton et al., 2022a; Stratton et al. 2022b; Stratton et al. In review). Some of these parasites are trophically transmitted (e.g. the *Ovipleistophora* in fish, trematodes and crayfish; Bojko et al. 2020a; Stratton et al. 2022a), where others are passed horizontally between crayfish. Species such as *Astathelohania* (=*Thelohania*) *contejeani* appear able to infect both native and invasive species, such as the likely parasite acquisition noted in signal crayfish in the UK (Anderson et al. 2021; Stratton et al. 2022b). In this study, we introduce another microsporidian species to the genus *Cambaraspora* – a genus currently known only to infect crayfish in the USA.

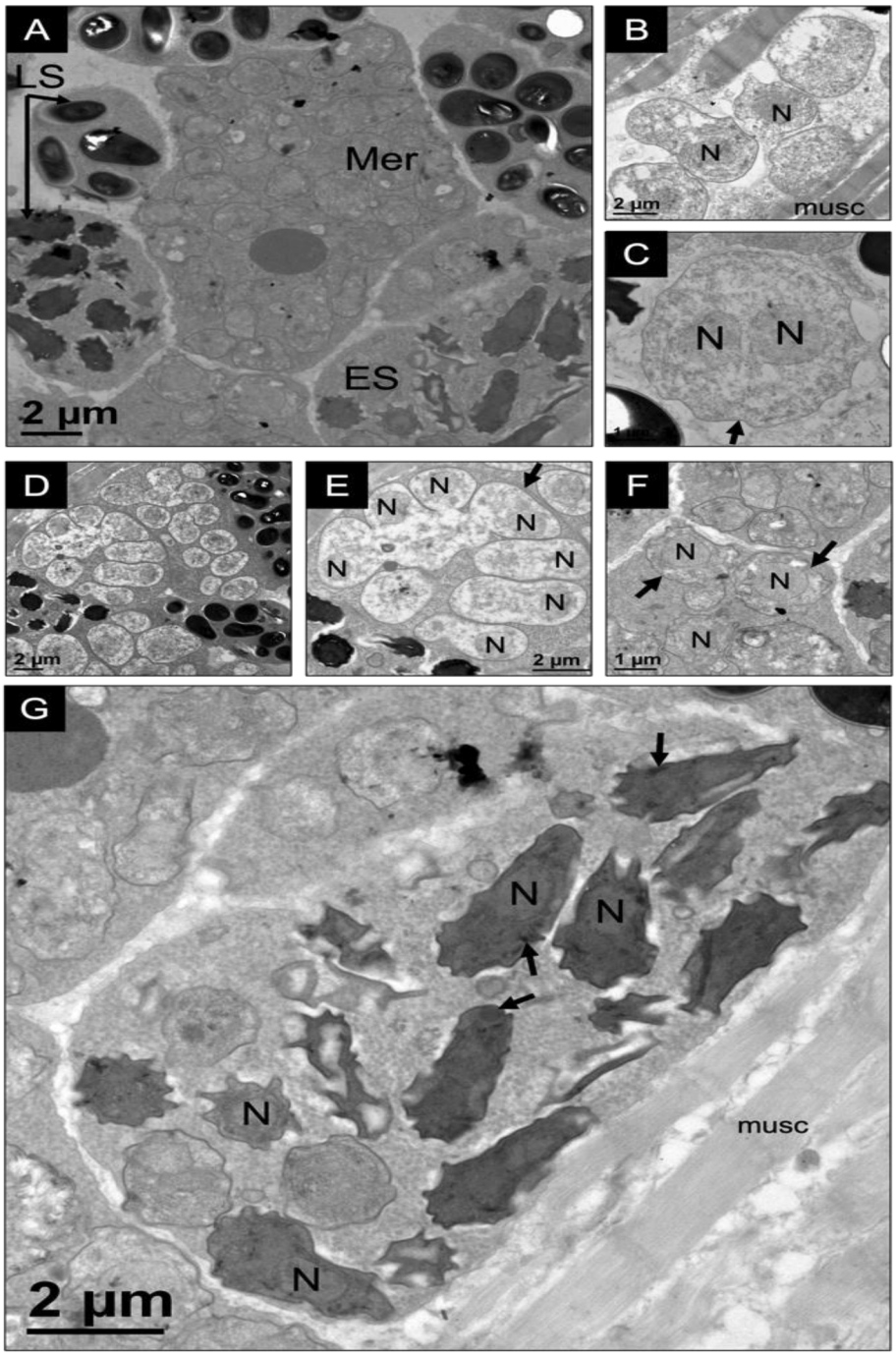

Fig. 2. Merogony and early sporogony of *Cambaraspora faxoni* n. sp. (A) This image highlights various stages developing within their own sporophorous vesicles (SPV) including a merogonal stage (Mer), early sporoblast stage (ES), and late sporoblast stage (LS). (B) Monokaryotic meronts (N = nucleus) developing in direct contact with host cytoplasm (musc = host muscle tissue). (C) Nucleus has divided to produce diplokaryotic (N = nucleus) meront. (D) Sporoginal plasmodia dividing to form sporonts. (E) A higher magnification image of a sporoginal plasmodium dividing to form up to 32 unikaryotic (N) sporonts within an SPV (arrow). (F) Unikaryotic sporonts with thickening plasmalemma (arrows). (G) As sporonts progress towards sporoblasts their plasmalemma becomes electron dense and they begin to develop electron dense organelles (arrows).

5.1. Evolutionary diversification of crayfish microsporidia

Microsporidia in crayfish are globally present with the two largest genera (*Astathelohania* and *Nosema*) found across continents (Moodie et al. 2003a; Moodie et al. 2003b; Moodie et al. 2003b; Pretto et al. 2018; Stratton et al. 2022b). For example, 5 *Astathelohania* spp. have been described, globally, from Australia, Europe, and North America (Moodie et al. 2003a; Moodie et al. 2003b; Pretto et al. 2018; Stratton et al. 2022b). A phylogenetic comparison suggested that host-parasite co-evolution may have occurred with this group of Microsporidia and their crayfish hosts (Stratton et al. 2022b). Similarly, crayfish-infecting members of the genus *Nosema* are found in Europe and Australia (Moodie et al. 2003c; Pretto et al. 2018). The *Ovipleistophora* is another globally distributed genus; however, crayfish-infecting members of this genus have only been found in the USA (Bojko et al. 2020a; Weng et al. 2021; Stratton et al. 2022a). Most recently, a novel member of the genus *Alternosema* was discovered in a North American crayfish host, with this entire genus restricted to North America (Lipa et al. 2020; Stratton et al. In review). Finally, the *Cambaraspora* genus appears restricted to crayfish hosts within the USA and was thought to be restricted to Florida (Bojko et al. 2020b); however, new information in this study suggests that *Cambaraspora* is distributed across the Eastern USA. The USA appears to be a center for crayfish-infecting microsporidian diversity.

North American crayfishes (Family Cambaridae) are hosts to the greatest diversity of microsporidian parasites globally, with over half of the formally described crayfish-infecting Microsporidia being found in the USA (Bojko et al. 2020a; Bojko et al. 2020b; Stratton et al. 2022b; Stratton et al. In review). The Cambaridae is the most diverse crayfish

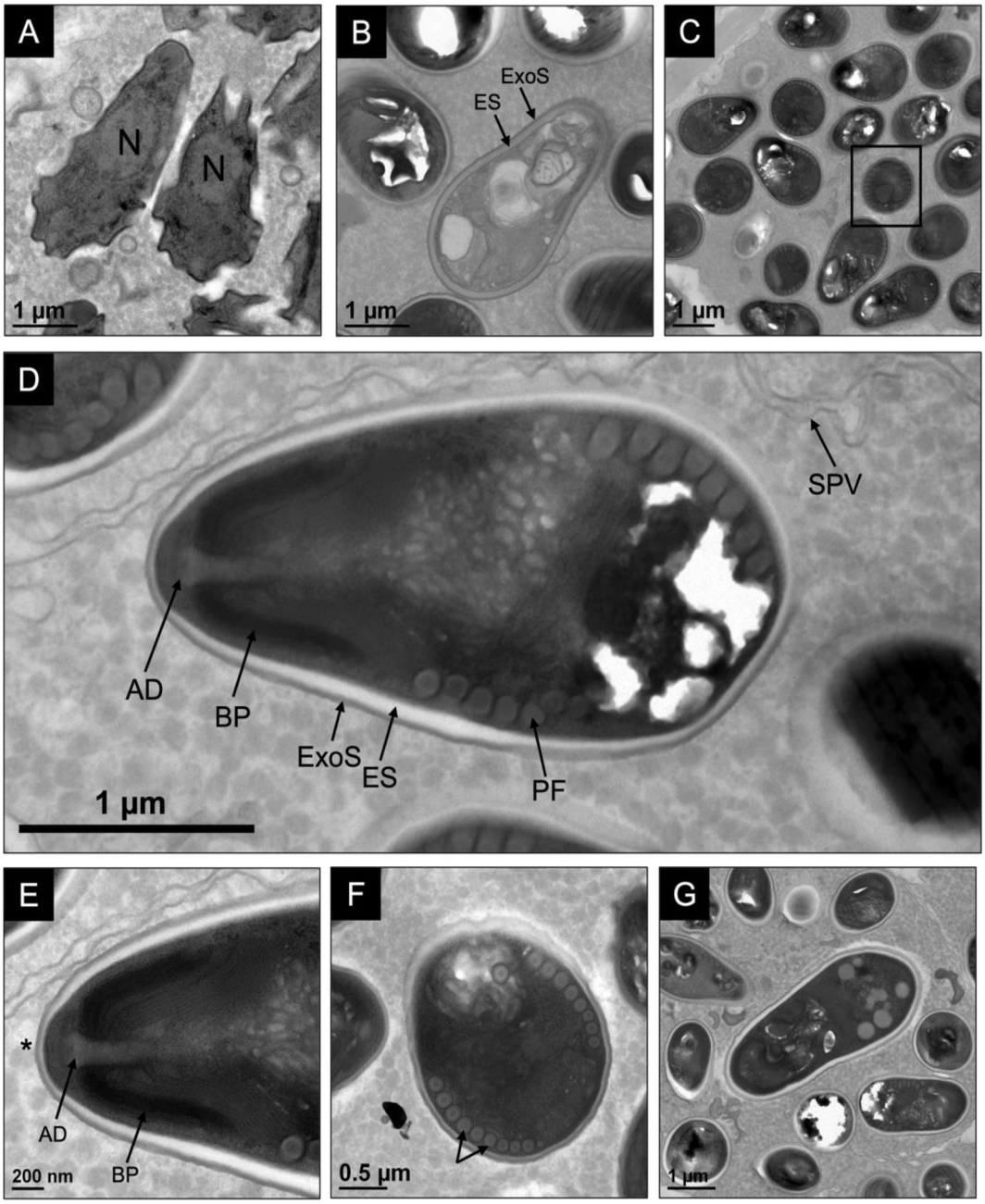

Fig. 3. Sporogony *of Cambaraspora faxoni* n. sp. (A) Two unikaryotic (N) sporoblasts developing within SPV. (B) An electron-lucent spore with a well-developed spore wall composed of an electron-lucent endospore (ES) and electron-dense exospore (ExoS). (C) Twenty spores observed developing within a single SPV. The highlighted spore illustrates the mature spore is diplokaryotic. (D) The ultrastructure of mature spores included an anchoring disk (AD), bi-layered polarplast (BP), polar filament (PF), and a spore wall composed of electron-lucent endospore (ES) and electron-dense exospore (ExoS). (E) Details of the anchoring disk-polar sac complex are highlighted including the bell-shaped anchoring disk (AD) and bi-layer polarplast (BP). The spore wall thins at the apex of the spore above the anchoring disk (*). (F) This image highlights the details of the anisofilar polar filament (arrows) including rings of protein. (G) Exhibits the morphological plasticity of mature spores.

lineage, housing two thirds of all extant crayfish species and is restricted to North America (Crandall and De Grave, 2017). Some Microsporidia have a narrow host specificity, typically only infecting a single host or several closely related hosts (Willis and Reinke, 2022). Therefore, it is likely North America will continue to have the greatest diversity of crayfish-infecting Microsporidia, and we are likely just beginning to understand the breadth of these parasites within the Cambaridae.

5.2. *Cambaraspora faxoni* – Invasive or native parasite?

Microsporidian parasite invasions have accompanied several crustacean invasions (Bojko and Stentiford, 2022). Several taxonomic lineages are implicated: *Hepatospora eriocheir* invaded with the Chinese mitten crab (*Eriocheir sinensis*) between China and the UK (Stentiford et al. 2011). *Cucumispora ornata* invaded with the demon shrimp (*Dikerogammarus haemobaphes*) between the Ponto-Caspian region and the UK (Bojko et al. 2015). *Fibrillanosema crangonycis* invaded with the freshwater amphipod *Crangonyx pseudogracilis*, between Europe and the UK (Slothouber Galbreath et al. 2004). The aforementioned species originate from different higher taxa: *H. eriocheir* (Enterocytozoonida); *C. ornata* (Glugeida); *F. crangonycis* (Neopereziida).

Some of the known invasive Microsporidia have been tied with

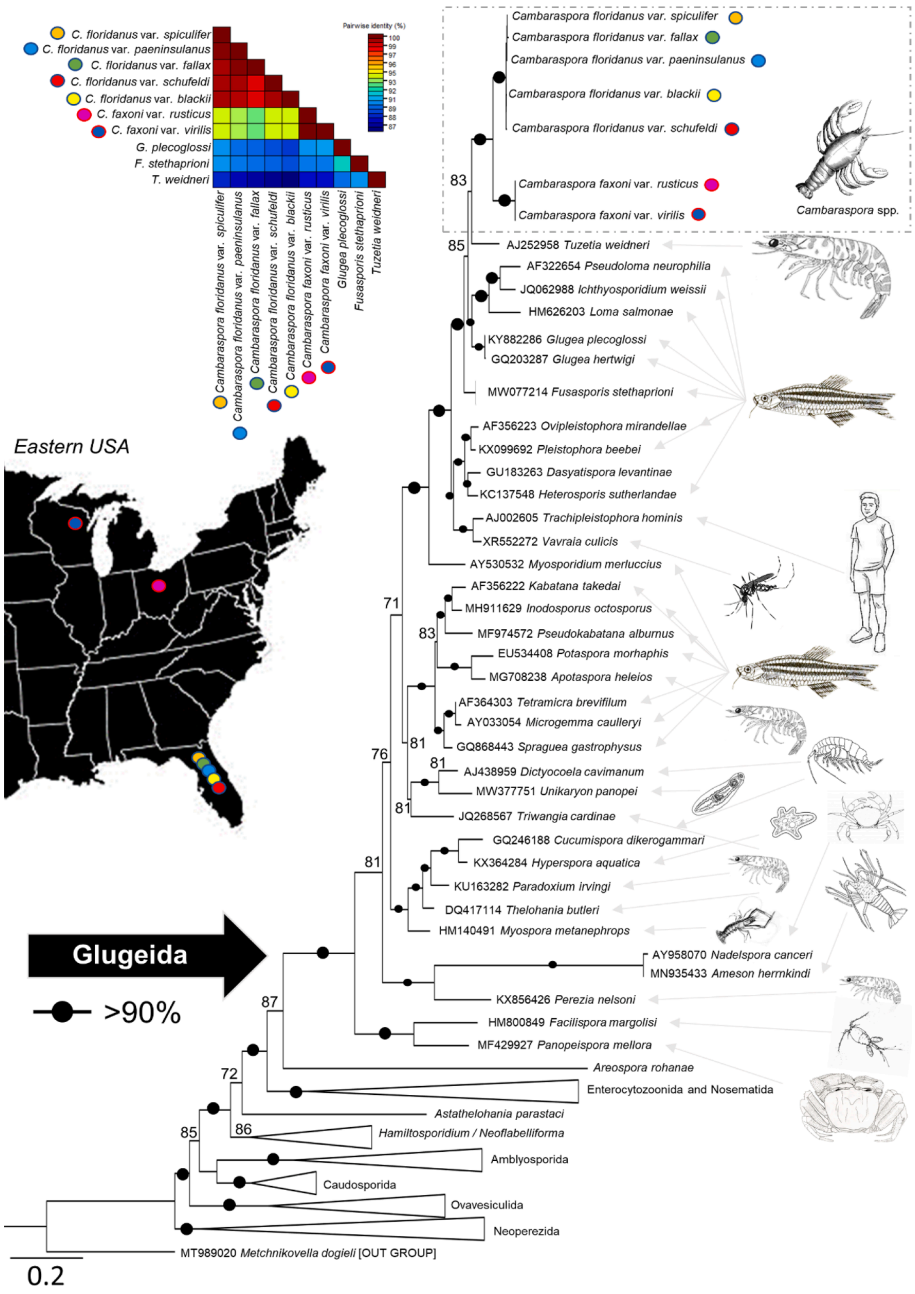

Fig. 4. A maximum-likelihood phylogenetic tree of all *Cambaraspora* isolates and 120 isolates representative of each major microsporidian group. A colored circle next to each *Cambaraspora* isolate corresponds to the annotated map indicating the location each isolate was found and to the isolates within the similarity matrix. The similarity matrix highlights the genetic similarity of the SSU gene within each *Cambaraspora* isolate and members of the Tuzetiidae and Glugeidae families.

changes to their host's invasion dynamic. *Cucumispora ornata* was found to decrease the demon shrimp's activity and lower its survival, suggesting that the parasite may regulate the impacts of the non-native amphipod (Bojko et al. 2019). However, this parasite poses a threat to a native amphipod, *Gammarus pulex*, and is spreading rapidly across the UK (Bojko et al. 2019; Burgess and Bojko, 2022). Another example includes *H. eriocheir,* which has been found to infect several hosts in the UK, and was likely to have been introduced by invasive mitten crabs (Bateman et al. 2016; Bojko and Stentiford, 2022).

Less is known regarding the ecological relationship between invasive crayfish and their microsporidian parasites. *Astathelohania contejeani* (Glugeida +) infects both the invasive signal crayfish (*Pacifastacus leniusculus*) and the native white clawed crayfish (*Austropotamobius pallipes*) within the UK, but the prevalence of this parasite is much higher in the endangered white clawed crayfish (Dunn et al. 2009). The high sequence similarity of isolates from these hosts, along with the historical range of the parasite, may suggest that the invasive signal crayfish acquired *A. contejeani* from the native white clawed crayfish (Anderson et al. 2021); however, the opposing argument that signal crayfish may have introduced this parasite from the USA is also plausible.

We found severe muscular infection and degeneration in *F. rusticus* and *F. virilis*, possibly relating to a loss of muscle function in the host. Similar microsporidian infections result in decreased host activity (Bojko et al. 2019). If crayfish hosts are impacted in a similar manner, *Faxonius* crayfish infected with *C. faxoni* may be less competitive. Therefore, this parasite might be relevant for crayfish invasion dynamics and interactions between *F. rusticus* and *F. virilis*.

In this study, we describe a new species of *Cambaraspora* found in both *F. rusticus* and *F. virilis. Faxonius rusticus* is native to the Ohio River drainage in southeastern Indiana, southwestern Ohio, and northern

Kentucky, USA (Taylor et al. 2015). *Faxonius virilis* is the most widely distributed crayfish in North America - found across much of the Midwest USA, but its native range does not overlap with the native range of *F. rusticus* (Thoma and Jezerinac 2000; Simon 2001; Taylor et al. 2015). Both species have been widely introduced outside of their native range across North America. In the upper Midwest USA, *F. rusticus* is a prolific invader that often replaces *F. virilis* in lakes and streams (Olden et al. 2006). *Faxonius virilis* has also been widely introduced across the USA, including some areas of the native range of *F. rusticus* in Indiana and Ohio (Thoma and Jezerinac 2000; Reisinger et al. 2020). The *C. faxoni* isolates infecting each host were 100% identical at the SSU. This host-parasite relationship may be explained by three hypotheses (Fig. 5):

(1) *Cambaraspora faxoni* is a co-invader and was introduced along with *F. rusticus* to Wisconsin, although we have not yet detected *C. faxoni* in Wisconsin *F. rusticus*. *Faxonius rusticus* has not been documented from the same site where we sampled the *F. virilis* collected in this study; however, it is present approximately three kilometers downstream with the populations separated by a small impoundment (Kabalan et al. unpublished data).

(2) *Cambaraspora faxoni* may be a native parasite to both hosts, with a broad geographic range, potentially co-evolving with both hosts in the lower Midwest USA where both species are present or perhaps distributed through feeding birds or a similar mechanism. Given that *Cambaraspora* spp. infect several crayfish hosts, and specific species (i.e., *C. floridanus*) can infect multiple crayfish species, a wide host range is predictable for *C. faxoni* (Bojko et al. 2020b).

(3) *Cambaraspora faxoni* infects an alternative host whose range overlaps with both crayfish hosts. This could include an intermediate host similar to what is found in some Amblyosporida species (Sweeney et al. 1985). Alternatively spores could be distributed via wading birds or a similar mechanism.

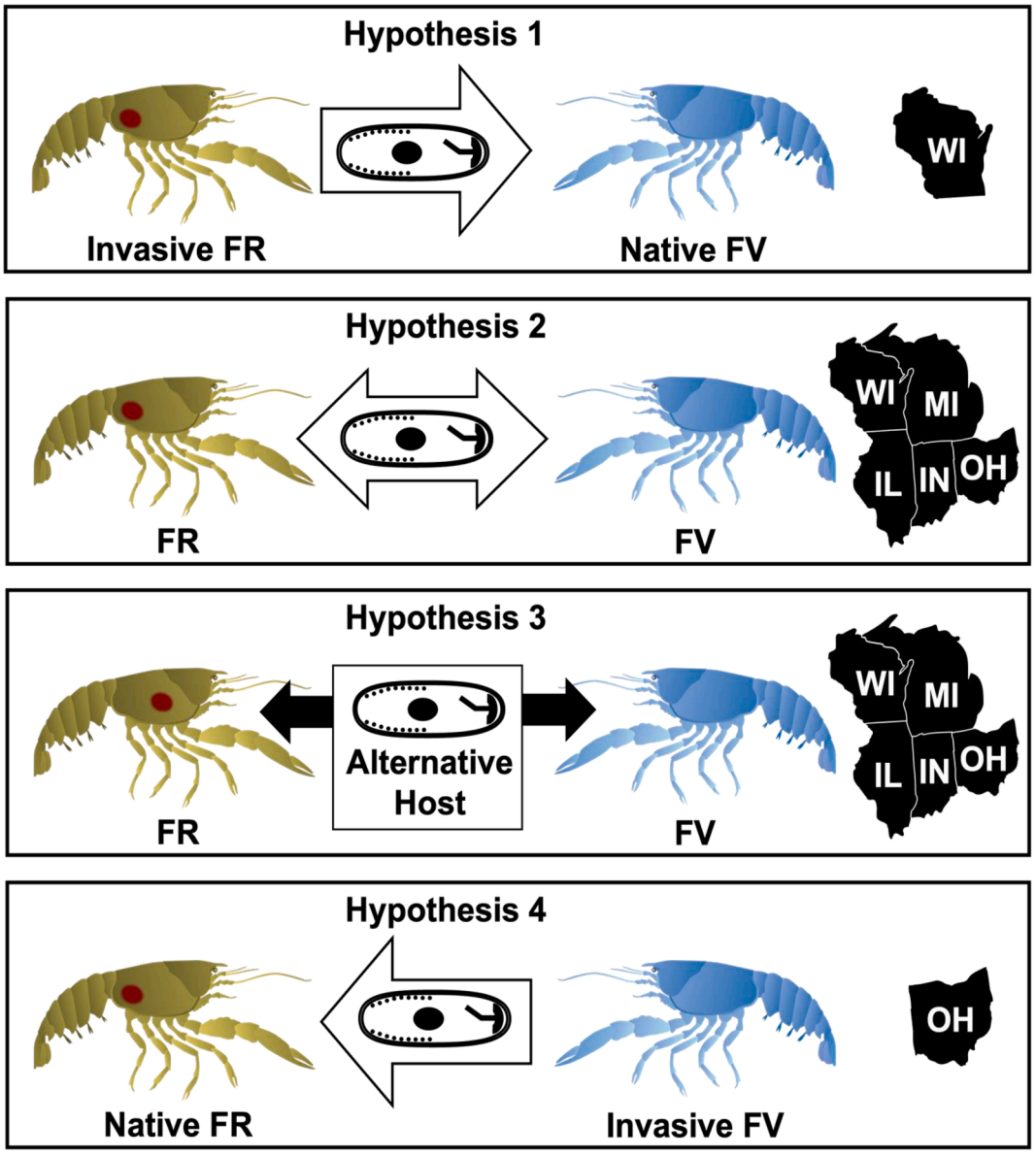

Fig. 5. Four hypotheses that may explain the host-parasite relationship observed between the microsporidium *Cambaraspora faxoni* n. sp. and it's two crayfish hosts, *Faxonius rusticus* (FR) and *Faxonius virilis* (FV). (1) *Faxonius rusticus* co-introduced *C. faxoni* to Wisconsin. (2) *Cambaraspora faxoni* has a broad geographic and host range and co-evolved with both hosts in the Midwest USA. (3) An alternative host for the microsporidium exists which transmits *C. faxoni* to both crayfish hosts. (4) *Faxonius virilis* co-introduced *C. faxoni* to Ohio. Crayfish image: Tracey Saxby, Integration and Application Network (ian.umces.edu/media-library).

(4) *Cambaraspora faxoni* was introduced to Ohio along with *F. virilis,* but *F. virilis* are not known from the drainage where we sampled the *F. rusticus* in our study making this hypothesis least likely.

5.3. Conclusion

Freshwater crayfishes have extensive ecological impacts and are one of the most widespread invasive groups in freshwater ecosystems (Twardochleb et al. 2013). Therefore, it is important to understand the parasites that both native and invasive crayfishes harbor. Here, we describe a novel member of the *Cambaraspora*, *C. faxoni* n. sp. (Glugeida: Tuzetiidae), infecting *F. virilis* and *F. rusticus* from Wisconsin and Ohio, respectively. We also broaden the known host range of *C. floridanus* to include *P. spiculifer* in Florida, another invasive host related to this microsporidian group.

Increased surveillance is necessary in both the native and invasive range of these species to understand the impact the parasite may have on invasion dynamics. Future studies should use histopathology and molecular diagnostics to determine the prevalence of this microsporidium in both ranges since individuals in this study only included overtly infected crayfish. In addition, genomic studies would be a valuable exploration tool, to determine how these *Cambaraspora* spp. infect such a wide range of crayfish hosts.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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