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Prevalence and molecular characterization of novel species of the Diplomonad genus *Octomitus* (Diplomonadida: Giardiinae) from wildlife in a New York watershed

Matthew H. Seabolt [a,b], Kerri A. Alderisio [c], Lihua Xiao [d,e], Dawn M. Roellig [a,*]

[a] Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, 30329, USA
[b] CFD Research Corporation, Huntsville, AL, 38506, USA
[c] Division of Water Quality Science and Research, Bureau of Water Supply, New York City Department of Environmental Protection, Valhalla, NY, USA
[d] Center for Emerging and Zoonotic Diseases, College of Veterinary Medicine, South China Agricultural University, Guangzhou, 510642, Guangdong, China
[e] Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou, Guangdong, 510642, China

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ABSTRACT

Octomitus is a diplomonad genus known to inhabit the intestinal tracts of rodents. Ultrastructural morphology and 18S rDNA gene sequence analysis support the placement of *Octomitus* as the closest sister lineage to *Giardia*, a parasite which causes diarrheal disease in humans and animals worldwide. However, further information on the ecology and diversity of *Octomitus* is currently scarce. Expanding the available database of characterized sequences for this organism would therefore be helpful to studies of Diplomonad ecology, evolution, and epidemiology, particularly related to the evolution of parasitism in *Giardia* and *Spironucleus,* another related Diplomonad common in commercial fish farming. In order to study the prevalence and genotypic diversity of *Octomitus*, we developed a nested PCR assay specific to *Octomitus* and optimized to detect genotypes in fecal samples collected from wildlife in a New York watershed, and sequenced a portion of the small subunit ribosomal DNA (18S rDNA) gene to identify samples to species level. Molecular evidence suggested that *Octomitus* genotypes display similar prevalence to *Cryptosporidium* and microsporidian pathogens in wildlife as well as strong host preference for rodent and opossum hosts. Phylogenetic analysis showed strong support for 14 *Octomitus* genotypes, 13 of these novel, and patterns of host-parasite co-evolution.

1. Introduction

Diplomonads are a ubiquitous group of flagellated protozoa, the majority of which are parasitic and found inhabiting the intestinal tract of animals (Brugerolle, 2000). The most well-studied species in this group belongs to the genus *Giardia*, one of the leading causative agents of diarrheal illness in humans and animals globally, as well as *Spironucleus*, responsible for high mortality in wild and commercially farmed fish (Williams and Lloyd, 2013). The genus *Octomitus* is a sister lineage to *Giardia* on the basis of ultrastructural morphology and phylogenetic analysis based on the 18S ribosomal DNA (18S rDNA) gene, and together the two genera comprise the subfamily Giardiinae (Brugerolle et al., 1974; Brugerolle, 1975; Siddall et al., 1992, 1993; Keeling and Brugerolle, 2006). Further data on the biology of *Octomitus* is lacking, including its potential impact on public health.

The presence and molecular characterization of potential zoonotic *Giardia* genotypes in wildlife has been commonly investigated (Mateo et al., 2017; Wait et al., 2017; Helmy et al., 2018; Hillman et al., 2019; Rivero et al., 2020; Zahedi et al., 2020, as examples). Such studies are helpful for identifying potential animal sources of contamination in drinking water catchments or untreated recreational water and describing the genetic diversity of these protozoa, with recent reports of novel species and genotypes in wildlife (Hillman et al., 2016; Wait et al., 2017; Helmy et al., 2018; Lyu et al., 2020). An investigation of *Giardia* in wild rodents in Germany reported the incidental detection of *Octomitus* using an off-label commercial *Cryptosporidium/Giardia* direct fluorescent assay (DFA) test and qPCR described by Verweij et al. (2003) (Helmy et al., 2018). Nonspecific detections are problematic because DFA microscopy is a standard method for detecting *Giardia* cysts in clinical samples, but off-label use for detecting *Giardia* in wildlife scats are

* Corresponding author.
E-mail address: iyd4@cdc.gov (D.M. Roellig).

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reported in (e.g.) Hillman et al. (2019) and Helmy et al. (2018) as salient examples. Thus, molecular methods are increasingly used to screen for *Giardia*. The 18S small subunit rDNA locus is most commonly targeted by PCR-based methods for *Giardia* since multiple copies in the nuclear genome yield the highest amplification success rate (Thompson and Ash, 2016). However, false-positive detection of *Giardia* has been revealed by subsequent sequencing of PCR amplicons, likely due to sequence similarity between *Giardia* and *Octomitus* at the primer binding sites utilized by the Verweij 18S qPCR and nested PCR (Hopkins et al., 1997; Appelbee et al., 2003; Helmy et al., 2018). Additionally, *Octomitus* is morphologically indistinguishable from the cysts of *Giardia*; the original description of the genus *Octomitus* was "almond shaped, 8–12 μm in length and 5–7 μm wide" cysts (Prowazek, 1904; Adam, 1991). Thus, there is a clear need for a reliable method to differentiate between the two genera for accurate identification and subsequent decision-making.

Using wildlife scat samples collected around a New York City water supply watershed as a study area, here we present the first report on the prevalence, host specificity, and genotypic diversity of *Octomitus* using a novel nested PCR assay designed to specifically amplify a portion of the 18S rDNA gene in *Octomitus*.

2. Materials and methods

2.1. Specimens

304 scat specimens were collected from 31 species of wildlife living in the New York City watershed by staff from the New York City Department of Environmental Protection (NYCDEP) as part of an ongoing watershed monitoring project in collaboration with the Centers for Disease Control and Prevention (CDC). All animals were identified to species except for deer mice (*Peromyscus* spp.). Collection of fecal samples employed a variety of direct and indirect sampling techniques. For live wildlife, direct observation of defecation immediately prior to sample collection ensured correct identification of animal sources to species level, except for deer mice (identified to genus as *Peromyscus* spp.). Additional details describing fecal sample collection are reported in Feng et al. (2007). Feces were shipped unpreserved in coolers to the CDC for DNA extraction and PCR.

2.2. Primer design, PCR, and sequencing

Nested PCR primers were designed by manually aligning the only full-length *Octomitus* 18S rDNA reference sequence (Genbank accession no. DQ366277; Keeling and Brugerolle, 2006) with available homologous sequences from *Giardia, Spironucleus, Enteromonas, Trimitus,* and *Hexamita* using Mega version 7.0.21 (Kumar et al., 2016). In total, 28 Diplomonad taxa were represented by 72 sequences in the alignment. *Octomitus*-specific primers Oct16f, GGTAGCATACGCTTMCCTCAAAG, and Oct885r, GTCCAAAGTCGGCATCGTTTAC, were designed for the primary nested reaction, with Oct64f, ACAAGCTTYTACGGCGAAACTG, and Oct817r, CTTCCCCGTCGATCAAGATC, designed for the secondary PCR reaction to amplify a 753 bp region of the 18S gene target.

Total DNA was extracted as described in Guo et al. (2014) using a Fast DNA spin kit for Soil (MP Biomedicals, Irvine, CA). Nested PCR was carried out using an Applied Biosystems (Foster City, CA) Proflex thermal cycler as follows: initial denaturation at 94°C for 5 min; 35 amplification cycles of 94°C for 45s, annealing at 59°C for 45s, extension at 72°C for 60s, and lastly, a final extension step at 72°C for 7 min. The primary PCR master mix consisted of 5 μL of 10× PCR Buffer (final concentration of 1×; GeneAmp PCR Buffer II, Applied Biosystems), 100 μM dNTPs, 400 ng/μL BSA, 2 mM $MgCl_2$, 0.75 U Promega GoTaq DNA polymerase, 250 nM each of primers, 2 μL of template DNA, plus molecular grade water for a final reaction volume of 50 μL. The secondary nested PCR master mix consisted of 29.85 μL molecular grade water, 5 μL of 10× PCR Buffer, 100 μM dNTPs, 500 nM of secondary primers, 2 mM $MgCl_2$, 0.75 U polymerase, and 2 μL of primary PCR template. All specimens tested by PCR were run in duplicate, with molecular grade water as negative controls in both reactions and an *Octomitus* positive control identified from an opossum. PCR results were visualized on a 1.5% agarose gel. PCR-positive samples were sequenced in both directions using secondary PCR primers and BigDye v3.1 dideoxy chemistry on an Applied Biosystems 3500 genetic analyzer. Control DNA from *Giardia duodenalis* assemblages and *G. microti* using the primers and PCR conditions described above did not show any positive amplification.

2.3. Phylogenetic analysis

Raw sequence reads were trimmed, assembled into contigs, and manually corrected using ChromasPro version 2.1.7 and exported in FASTA format. Sequences were aligned by eye to the *Octomitus* reference sequence from Keeling and Brugerolle (2006) to generate an alignment containing only *Octomitus* sequences. The best-fitting substitution model for the final alignment was estimated using jModelTest2 using the Akaike information criteria (AIC) (Guindon and Gascuel, 2003). A maximum likelihood phylogeny was computed using PhyML v20130103 with the chosen evolutionary model and the best starting tree from jModelTest2 (Darriba et al., 2012). Statistical branch support was estimated using 1000 bootstrap replicates. The ML tree was visualized using the Interactive Tree of Life (iTOL) web portal (Letunic and Bork, 2019; URL: https://itol.embl.de). Additionally, to confirm the monophyly of *Octomitus* within the diplomonads, the *Octomitus* sequences were aligned against related diplomonad taxa as outgroups and analyzed using the same ML approach described above.

3. Results

3.1. Prevalence of Octomitus in wildlife

We detected *Octomitus* genotypes in 74 (24.3%) out of the 304 wildlife samples tested by nested PCR (Table 1). All positive samples originated from the rodent families Cricetidae (containing deer mice and voles; 45/101), Scuiridae (squirrels; 4/46), Muridae (murine rodents; 13/33), or from the Virginia opossum, the sole marsupial species in North America (11/19, 57.9%). *Octomitus* was not detected in specimens from any other sampled vertebrate host taxa; however the sample size of insectivores, ruminants, lagomorphs, birds, and amphibians is too small (n = 1–9) to draw accurate estimates of *Octomitus* infection, if they are present in these hosts.

3.2. Octomitus genotypes in wildlife

Sequences of 74 PCR-positive specimens were aligned against the available reference sequence of *Octomitus intestinalis* (Keeling and Brugerolle, 2006) for species and genotype determination. None of the sequences matched this reference with 100% nucleotide identity, thus they were designated as novel genotypes and named in sequential order by the host in which the genotype was first identified (e.g. opossum genotype I, II, etc.). Within *Octomitus*, the average nucleotide identity between non-redundant pairs of sequences was 97.00%, ranging from 95.74% to 99.86%, which equated to an average of 15 SNPs. *Octomitus* sequences shared an average identity of 80.8% (=35 SNPs) with the homologous region of *Giardia*. In total, 13 novel genotypes were identified and summarized in Table 2. Five genotypes (deer mouse genotypes I, II, and III; opossum genotypes I and II) were identified in at least two host species each, with the house mouse (*Mus musculus*), deer mice (*Peromyscus* spp.), opossum (*Didelphicus virginiana*), and eastern chipmunk (*Tamias striatus*) identified most commonly. The remaining genotypes were observed in a single host species. Some host species were found to host multiple unique genotypes of *Octomitus*, namely the house mouse (5 genotypes); deer mice (4 genotypes); opossum, eastern chipmunk, meadow voles (3 genotypes each); Norway rat and boreal red-backed vole (2 genotypes each). No samples were found to have

Table 1
Prevalence of *Octomitus* genotypes in wildlife from the NYCDEP watershed.

Host	Total no. of samples	No. of positive samples	Prevalence (%)	Genotypes (no. positive)
Rodents	200	63	31.5	
Sciuridae	46	5	10.9	
Sciurus carolinensis (eastern grey squirrel)	31	1	3.2	Opossum genotype I (1)
Sciurus vulgaris (red squirrel)	2	0	0.0	
Glaucomys volans (southern flying squirrel)	1	0	0.0	
Tamias striatus (eastern chipmunk)	7	3	42.9	Deer mouse genotype I (1), deer mouse genotype II (1), deer mouse genotype III (1)
Marmota monax (woodchuck)	5	1	20.0	Woodchuck genotype (1)
Castoridae	15	0	0.0	
Castor canadensis (beaver)	15	0	0.0	
Erethizontidae	4	0	0.0	
Erethizon dorsatum (North American porcupine)	4	0	0.0	
Muridae	33	13	39.4	
Rattus norvegicus (Norway rat)	7	2	28.6	Rat genotype (1), deer mouse genotype V (1)
Mus musculus (house mouse)	26	11	42.3	Mouse genotype I (1), mouse genotype II (6), opossum genotype I (1), deer mouse genotype I (1), deer mouse genotype II (2)
Zapodidae	1	0	0.0	
Napaeozapus insignis (woodland jumping mouse)	1	0	0.0	
Cricetidae	101	45	44.6	
Peromyscus sp. (deer and wood mice)	85	39	45.9	Deer mouse genotype I (22), deer mouse genotype II (15), deer mouse genotype III (1), deer mouse genotype IV (1)
Myodes gapperi (boreal red-backed vole)	5	2	40.0	Vole genotype I (1), Vole genotype III (1)
Microtus pennsylvanicus (meadow vole)	10	4	40.0	Vole genotype II (2), deer mouse genotype I (1), opossum genotype II (1)
Ondatrini zibethicus (muskrat)	1	0	0.0	
Carnivores	61	0	0.0	
Mustela vison (mink)	6	0	0.0	
Mustela erminea (ermine)	1	0	0.0	
	30	0	0.0	
Procyon lotor (raccoon)				
Ursus americanus (black bear)	5	0	0.0	
Lontra canadensis (river otter)	8	0	0.0	
Mephitis mephitis (striped skunk)	10	0	0.0	
Felis cattus (domestic cat)	1	0	0.0	
Insectivores	5	0	0.0	
Blarina brevicauda (northern short-tailed shrew)	5	0	0.0	
Lagomorpha	9	0	0.0	
Sylvilagus floridanus (eastern cottontail)	9	0	0.0	
Ruminants	3	0	0.0	
Odocoileus virginianus (white-tailed deer)	3	0	0.0	
Marsupials	19	11	57.9	
Didelphis virginiana (Virginia opossum)	19	11	57.9	Opossum genotype I (9), opossum genotype II (1), deer mouse genotype I (1)
Birds	6	0	0.0	
Branta canadensis (Canada goose)	2	0	0.0	
Melospiza melodia (song sparrow)	1	0	0.0	
Actitis macularius (spotted sandpiper)	1	0	0.0	
Oxyura jamaicensis (ruddy duck)	1	0	0.0	
Tachycineta bicolor (tree swallow)	1	0	0.0	
Amphibians	1	0	0.0	
Bufo americanus (American toad)	1	0	0.0	
Total	304	74	24.3	

mixed genotypes, which would have appeared as double peaks or background signals in the sequence chromatograms.

3.3. Phylogenetic relationships among Octomitus genotypes

The final trimmed sequence alignment contained 74 sequences covering a 723 bp region of the 18S rDNA gene, including several variable regions. Maximum likelihood analysis confirmed genotype identification and estimated evolutionary relationships among genotypes. The resulting ML phylogeny is shown in Fig. 1. The tree was rooted using the most divergent sequence, deer mouse genotype IV. Two distinct sequence variants (n = 12 and 15) of deer mouse genotype I were observed in the alignment, comprising one thymine insertion and one T->C transition. While these mutations are consistent at the same sites

Table 2
Animal hosts identified in this study.

Novel *Octomitus* genotype	Hosts identified in this study (no. positive samples)
Deer mouse genotype I	Deer mouse (22), eastern chipmunk (1), meadow vole (1), Norway rat (1), opossum (1), house mouse (1)
Deer mouse genotype II	Deer mouse (15), house mouse (2), eastern chipmunk (1)
Deer mouse genotype III	Deer mouse (1), eastern chipmunk (1)
Deer mouse genotype IV	Deer mouse (1)
Opossum genotype I	Opossum (9), house mouse (1), eastern grey squirrel (1)
Opossum genotype II	Opossum (1), meadow vole (1)
Vole genotype I	Boreal red-backed vole (1)
Vole genotype II	Meadow vole (2)
Vole genotype III	Boreal red-backed vole (1)
Mouse genotype I	House mouse (1)
Mouse genotype II	House mouse (6)
Rat genotype	Norway rat (1)
Woodchuck genotype	Woodchuck (1)

* all genotypes here are novel and phylogenetically distinct from published *O. intestinalis* sequences (Keeling et al., 2006; Helmy et al., 2018).

across samples in the alignment, these were considered the same genotype by bootstrap resampling in our phylogenetic analyses. Thus, the ML phylogeny confirmed 13 unique lineages with good branch support. Collapsing nodes with low statistical support revealed a polytomic node towards the base of the tree, from which arose four lineages: opossum genotypes I and II, a lineage containing the woodchuck genotype and deer mouse genotype III as sister taxa, and lastly, a lineage containing all remaining sequences, which are all from rodents with the exception of a single opossum host. Within this fourth lineage, a branch from murine hosts (the Norway rat and house mouse) is well supported, which incidentally includes the only sequence of vole genotype II as well as the *Octomitus intestinalis* reference sequence. Vole genotypes I and III clustered together as a distinct lineage, sister to a lineage containing the two most commonly identified genotypes (deer mouse genotypes I and II). ML analysis of the broader diplomonad alignment confirmed that the genotypes identified here form a well-supported monophyletic clade within the Diplomonads and confirmed the genus *Octomitus* as a sister lineage to *Giardia* (Fig. 1, inset).

4. Discussion

This study is the first to introduce an assay specific to *Octomitus* for molecular detection and characterization of genotypes. The development of a robust molecular detection assay for this organism is of interest due to the close evolutionary relationship between *Octomitus* and *Giardia*, the latter being a parasite of major public health concern in humans and animals. Here, we report the identification of 13 novel genotypes from New York City watersheds based on sequence analysis of 723 bp of the 18S rDNA gene, which constitutes the first dataset on the prevalence of *Octomitus* in wildlife. Our data revealed *Octomitus* isolated from eight species from three rodent families and the North American opossum as hosts, the latter being a newly identified host for this genus. Overall, the prevalence of *Octomitus* in 304 wildlife scat samples tested is approximately 24.3%, comparable to previous results reporting on prevalence of *Enterocytozoon bienusi* and *Cryptosporidium* in the same New York City watersheds (29.0% and 20.5% respectively; Guo et al., 2014; Feng et al., 2007).

Wildlife species with larger sample sizes tended to host multiple *Octomitus* genotypes, which suggests that larger sample sizes would continue to reveal novel parasite diversity. Additionally, these data support the pattern of host-parasite co-evolution, as *Octomitus* genotypes which were more closely related to each other were often identified in hosts that also share evolutionary relatedness. For example, vole genotypes I and III appeared as sister lineages on the tree in Fig. 1, and both were uniquely identified in boreal red-backed voles. The rat genotype and mouse genotypes (from murine hosts) also clustered together with the *O. intestinalis* reference sequence from a wild mouse (Keeling and Brugerolle, 2006). Notably, this lineage also includes vole genotype II, identified in two meadow voles (Cricetidae). The two marsupial genotypes, opossum genotypes I and II, likewise branched from the same node towards the base of the tree. Deer mouse genotypes III and IV, which were found only twice and once, respectively, did not cluster with the other two deer mouse genotypes identified in this study. In fact, deer mouse genotype IV appears external to all other genotypes, which may be a long-branch artefact pulling it towards the base of the tree due to substantial insertions in the sequence. Interestingly, Helmy et al. (2018), while investigating the prevalence of *Giardia* in wild rodents in Germany, identified three short fragments (approx. 250 bp) of the 18S rDNA gene as *Octomitus*. While we did not include these sequences in our phylogenetic analysis due to the difference in amplicon size from our data, these sequences are recognizably unique from one another and the *O. intestinalis* reference sequence, which confirms the presence of *Octomitus* in European wildlife and suggests similar patterns of genotype diversity as we identified in New York.

It is currently unknown if any *Octomitus* genotypes have zoonotic potential as human or veterinary pathogens, as data on the life history of *Octomitus* is scarce, including experimental information on the cross-reactivity of *Octomitus* genotypes with assays designed to detect *Giardia*. Our results plus the information reported by Helmy et al. (2018) suggest that *Octomitus* genotypes exhibit moderate to strong host preference in nature; however it is noteworthy that the hosts identified in this study, rodents and opossums, frequently come into contact with humans as household pests. Further, small sample sizes in non-rodent host taxa are a substantial limitation and may have hindered the detection of further genotypes. Based on these data and similar findings in other protozoan genera represented by a mixture of human-infectious and wildlife genotypes (Guo et al., 2014; Zahedi et al., 2016; Helmy et al., 2018; Li et al., 2019), it seems unlikely that *Octomitus* is of public health significance as a zoonotic pathogen. However, the absence of such data is strongly attributable to the lack of a robust detection method and thus remains to be confirmed experimentally. In addition, it remains unknown how frequently genotypes of *Octomitus* may co-occur with known human pathogens such as *Giardia*, *Cryptosporidium*, or microsporidia. Prior to the genotypes identified here being elevated to novel species of *Octomitus*, more data should be gathered characterizing their genetic relatedness at additional loci, ecology (e.g. host preference), and morphology to better assess evolutionary relationships. These new data presented here should be helpful to future studies analyzing the prevalence, diversity, and evolution of Diplomonad species. More studies evaluating the occurrence and distribution of *Octomitus* species are needed for a better understanding of this parasite's biology and potential public health significance, if any, to emerge.

5. Conclusions

The present study characterized novel genotypes of *Octomitus* using a newly developed PCR assay for molecular detection and genotyping. Our report expands the diversity and known host range of *Octomitus* and suggests that *Octomitus* genotypes occur with equal frequency as *Cryptosporidium* and microsporidia pathogens in wildlife reservoir hosts, particularly wild rodents and opossums, which are common in urban and suburban environments in North America. The reported data argue that *Octomitus* genotypes are generally host-adapted and likely pose little, if any, public health significance to humans, although this hypothesis remains to be experimentally demonstrated in the future.

Disclaimers

The findings and conclusions in this report are those of the authors

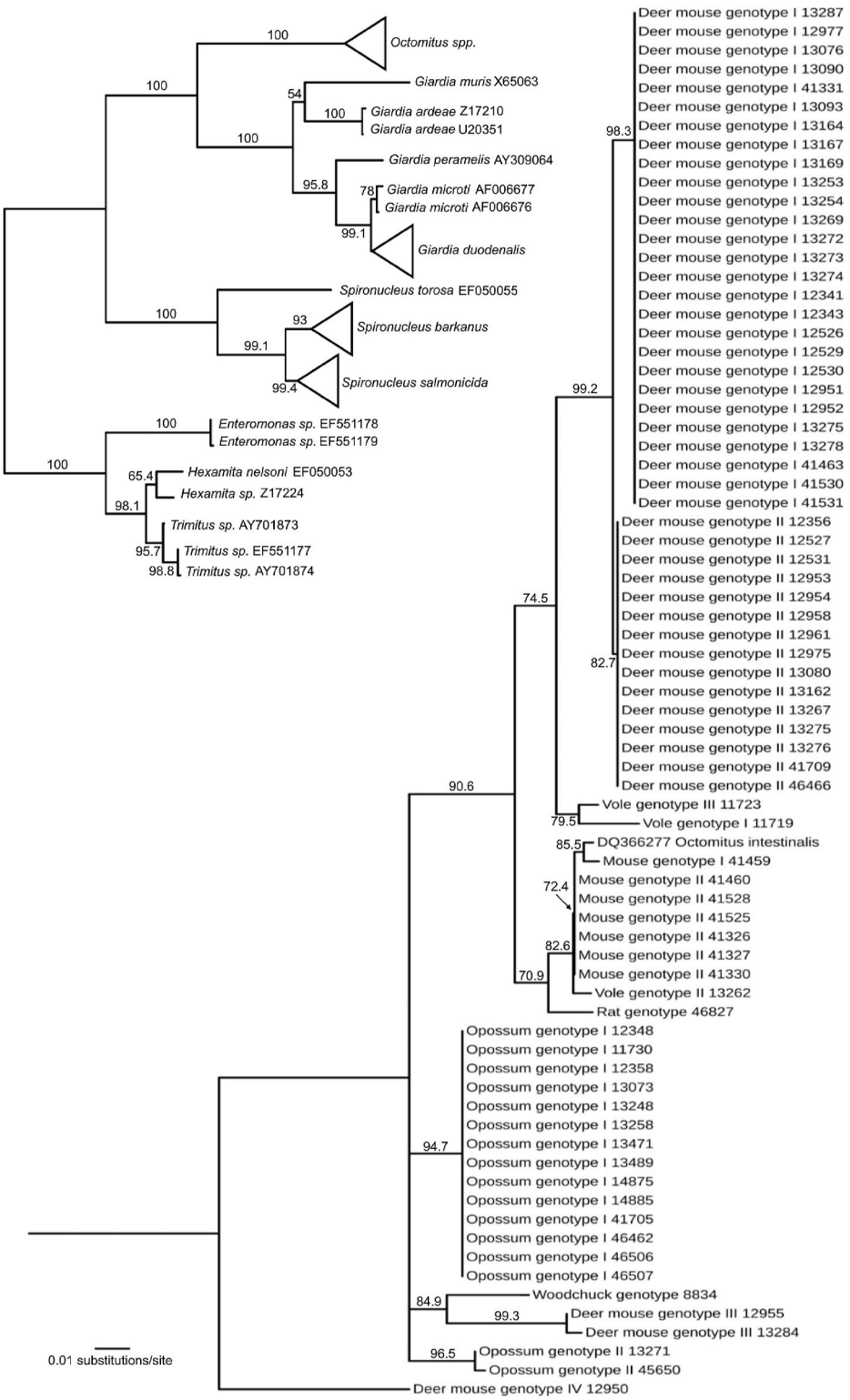

Fig. 1. Phylogenetic relationships between 74 sequences of *Octomitus* representing 14 genotypes estimated by maximum likelihood analysis. The GTR + I + G model (gamma shape = 0.338, prop. invariable sites = 0.619) was chosen by jModelTest2 to be the best-fitting evolutionary model. Branches with less than 70% bootstrap support were not considered statistically robust and were collapsed during manual editing of the visualization. **Inset**: ML phylogeny computed from *Octomitus* genotypes aligned with the homologous region of available Diplomonad 18S rDNA sequences from *Giardia*, *Spironucleus, Hexamita, Trimitus,* and *Enteromonas*.

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Data availability

Sequences generated as part of this study have been submitted to GenBank and are available as accession numbers MW289247 - MW289319.

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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