

## Nematodes and trematodes associated with terrestrial gastropods in Nottingham, England

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## Research Paper

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**Abstract**

A parasitological survey of terrestrial slugs and snails was conducted at popular dog walking locations across the city of Nottingham, with the intentions of finding gastropods infected with parasites of medical (or veterinary) importance such as lungworm (metastrongyloid nematodes) and trematodes. A total of 800 gastropods were collected from 16 sites over a 225 km<sup>2</sup> area. The extracted nematodes and trematodes were identified by molecular barcoding. Of the 800 gastropods collected, 227 were infected (172 had nematode infections, 37 had trematode infections and 18 had both nematode and trematode infections). Of the nematode infected gastropods genotyped, seven species were identified, *Agfa flexilis*, *Angiostoma gandavense*, *Angiostoma margaretae*, *Cosmocerca longicauda*, *Phasmarhabditis hermaphrodita*, *Phasmarhabditis neopapillosa* and an unknown Cosmocercidae species. Of the trematode infected gastropods genotyped, four species were identified, *Brachylaima arcuate*, *Brachylaima fuscata*, *Brachylaima mesostoma* and an unknown Plagiorchioidea species. No lungworm species were found within the city of Nottingham. To our knowledge, this study represents the first survey of gastropod-associated nematodes and trematodes in the East Midlands of the United Kingdom.

**Introduction**

Slugs and snails (Class: Gastropoda) comprise approximately 35,000 extant species and can host a diverse range of metazoan parasites (and parasitoids) such as cestodes, trematodes, nematodes, insects and acarids (Barker & Efford, 2004; Chapman, 2009). There are approximately 25,000 extant species of nematodes, of which 3500 are parasites of invertebrates (Grewal *et al.*, 2003). Of these, 50 metastrongyloid (lungworms) species are of medical or veterinary importance, with notable genera being *Aelurostrongylus*, *Angiostrongylus*, *Crenosoma*, *Elaphostrongylus*, *Muellerius*, *Neostrongylus*, *Oslerus*, *Prostrongylus* and *Troglostrongylus* (Alicata, 1965; Skorpning *et al.*, 1980; Campbell *et al.*, 1988; Diez-Baños *et al.*, 1989; Schjetlein *et al.*, 1995; Majoros *et al.*, 2010; Panayotova-Pencheva, 2011; Kim *et al.*, 2014; Patel *et al.*, 2014; Conboy, 2015; Helm *et al.*, 2015; Aziz *et al.*, 2016; Hadi, 2018; Hicklenton & Betson, 2019; Penagos-Tabares *et al.*, 2020). Nematodes have evolved diverse relationships with gastropods, with some species using them as an intermediate host (e.g. juveniles of lungworm species) while others (Rhabditidae, Mermithidae and Ascarididae) parasitize gastropods and use them as their definitive host; or for other means such as necromeny or transportation (paratenic) (Grewal *et al.*, 2003; Ivanova *et al.*, 2019).

Digenetic trematodes comprise approximately 40,000 extant species, with more than 18,000 described species (Cribb *et al.*, 2001; Kostadinova & Pérez-del-Olmo, 2014). Unlike nematodes, digenetic trematodes use invertebrates exclusively as an intermediate host, with a vertebrate (typically a fish, mammal, or bird) being used as their definitive host (Barker & Efford, 2004). Notable genera of medical or veterinary importance are *Clonorchis*, *Fasciola*, *Fasciolopsis*, *Gastrodiscoides*, *Heterophyes*, *Metagonimus*, *Opisthorchis*, *Paragonimus* and *Schistosoma* (Doughty, 1996; Kostadinova & Pérez-del-Olmo, 2014). Trematode species which infect terrestrial gastropods use them in order to infect bird, mammal, or reptile definitive hosts which prey on gastropods (Morley & Lewis, 2008). Most species specialize in infecting one type of definitive host, but some species can infect multiple hosts (Butcher & Grove, 2005). The lifecycle of these trematodes first involves a gastropod host being infected through the ingestion of eggs (excreted by an infected definitive host). After ingestion, it takes one to three months for asexual sporocysts to produce cercariae within the first intermediate gastropod host (Butcher & Grove, 2003). Gastropods can act as both the first and second intermediate host, as infected snails (first intermediate) shed cercariae in their mucus which can infect other gastropods through bodily contact (or themselves making them a first and second intermediate host simultaneously) (Butcher & Grove, 2005). The successful cercariae develop into mature metacercariae after four months and can survive up to another four months within the

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gastropod host. The transmission cycle is completed when the secondary intermediate gastropod host is ingested by a bird, mammal, or reptile definitive host (Morley & Lewis, 2008).

The current understanding of nematodes and trematodes associated with terrestrial gastropods in Europe is based on parasitological surveys conducted in Austria (Penagos-Tabares *et al.*, 2020), Belgium (Singh *et al.*, 2020), Bulgaria (and Crimea) (Ivanova *et al.*, 2013), the Czech Republic (Heneberg *et al.*, 2016), Denmark (Taubert *et al.*, 2009), England (Morley & Lewis, 2008; Patel *et al.*, 2014; Hicklenton & Betson, 2019), France (Nguyen *et al.*, 2017), Germany (Ross *et al.*, 2016; Lange *et al.*, 2018; Gérard *et al.*, 2020), Hungary (Majoros *et al.*, 2010), the Netherlands and Norway (Antzée-Hyllseth *et al.*, 2020), Poland (Filipiak *et al.*, 2020), Italy (Ivanova *et al.*, 2019), Slovenia (Laznik *et al.*, 2010), Scotland (Helm *et al.*, 2015), Spain (Foronda *et al.*, 2010; Jefferies *et al.*, 2010; Paredes-Esquivel *et al.*, 2019; Martín-Carrillo *et al.*, 2021) and Wales (Ross *et al.*, 2010a, b; Aziz *et al.*, 2016). The majority of these studies found no medically important nematode or trematode species, with primarily free-living, gastropod-specific and veterinary important species being reported. Four common lungworm genera (Metastrongyloidea) of medical/veterinary importance were present in Europe (*Angiostrongylus*, *Crenosoma*, *Aelurostrongylus* and *Troglostrongylus*) with *Angiostrongylus* (*An.*) *cantonensis* the only medically important species reported. *Angiostrongylus* (*An.*) *cantonensis* is a parasite endemic to Asia, the Caribbean and Pacific Islands. In Europe it has been found infecting black rats (*Rattus rattus*) in the Canary and Balearic Islands and the Algerian hedgehog (*Atelerix algirus*) in mainland Spain (Foronda *et al.*, 2010; Paredes-Esquivel *et al.*, 2019; Martín-Carrillo *et al.*, 2021). Furthermore, Nguyen *et al.* (2017) reported the first autochthonous human case of *An. cantonensis* infection in France. In addition to the metastrongyloids, seven additional gastropod-related nematode families were reported in Europe, the Agfidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogasteridae, Mermithidae and Rhabditidae. The most common genera of trematodes found were *Brachylaima*, *Eurytrema*, *Michajlovia*, *Urogonimus* and *Urotocus*. Certain species of *Brachylaima* (Brachylaimiasis) and *Eurytrema* (Eurytrematosis) have been found to cause infection within humans in Australia and Brazil, respectively (Schwartz *et al.*, 2015; Gracenea & Gállego, 2017) though there have as yet been no reports of human infection in Europe. Trematodes associated with terrestrial gastropods in Europe have not been as well studied as nematodes, most probably due to the majority of these species of medical or veterinary importance being associated with aquatic snail species.

Lungworm nematode infections have been extensively studied in Europe (Taubert *et al.*, 2009; Patel *et al.*, 2014; Helm *et al.*, 2015; Taylor, 2015; Aziz *et al.*, 2016; Helm & Morgan, 2017; Lange *et al.*, 2018; Elsheikha *et al.*, 2019; Hicklenton & Betson, 2019; Fuehrer *et al.*, 2020; Penagos-Tabares *et al.*, 2020). Lungworm infections are fatal to companion animals due to the severe respiratory disease and bleeding disorders caused by the parasite (Taubert *et al.*, 2009). *Angiostrongylus* (*An.*) *vasorum* and *Crenosoma vulpis* are widespread across the United Kingdom, with domesticated dogs and red foxes (*Vulpes vulpes*) acting as their definitive hosts (Helm & Morgan, 2017). Geography is one of the main risk factors for *An. vasorum* infections in dogs, with the most endemic areas of the United Kingdom being Southern England and Southern Wales (Patel *et al.*, 2014; Helm & Morgan, 2017; Hicklenton & Betson, 2019)

though *An. vasorum* in the United Kingdom is spreading northwards, with the parasite already established in Northern England and Scotland (Helm *et al.*, 2015; Aziz *et al.*, 2016). Reasons for the spread of *An. vasorum* include a warmer climate which favours the parasites' development and the urbanization of wild red fox populations acting as a reservoir of infection, with an estimated one in five infected (Taylor, 2015; Helm & Morgan, 2017). *Crenosoma vulpis* transmission is similar to *An. vasorum* but is more commonly reported in wild canid species than domesticated dogs (Lange *et al.*, 2018). Similarly, *Aelurostrongylus* (*Ae.*) *abstrusus* is a globally distributed lungworm species that infects wild and domesticated cat species, with a prevalence of 1.7% in United Kingdom house cats (Helm & Morgan, 2017; Elsheikha *et al.*, 2019). Lungworm infections in domesticated cats and dogs are thought to be underreported as some infections can be asymptomatic and milder cases are commonly misdiagnosed as other disorders such as hypersensitivity (Wright, 2009; Penagos-Tabares *et al.*, 2018; Pohly *et al.*, 2022).

The primary aim of this study was to investigate which species of terrestrial gastropods are commonly found at dog walking sites in the city of Nottingham and the county of Nottinghamshire, to determine which nematode and trematode species are associated with these gastropods and to determine infection rates. The secondary aim was to investigate whether lungworm nematode species that cause veterinary disease are found at popular dog walking sites across the city of Nottingham and the county of Nottinghamshire.

## Materials and methods

### Collection sites and gastropod identification

Slugs and snails were collected from 16 sites across Nottinghamshire from June to November 2020 and June to November 2021. All sites were popular dog walking locations and included recreational grounds, country parks, public gardens and nature reserves (table 1; fig. 1). A total of 800 gastropods were collected by hand with 50 specimens collected from each site and with a maximum of ten individuals per species being taken. Specimens were identified morphologically using a Terrestrial Mollusc Key (<https://idtools.org/id/mollusc/key.php>) (White-McLean, 2011) and the *Slugs of Britain and Ireland* as an illustrated guide (Rowson *et al.*, 2014).

### Gastropod dissection

Specimens were dissected into four equal pieces within 24-h of collection and placed into a 50 ml falcon tube containing Ash's digestion solution (0.7% pepsin in 0.5% hydrochloric acid) for four to eight hours (Ash, 1970). The solution was then placed into a 9 cm Petri dish and examined under a dissection microscope for the presence of nematodes, or the metacercariae stage of trematodes. Nematodes were categorized as either juvenile or adult worms. When found, nematodes and metacercariae were individually picked and placed into 0.2 ml tubes containing 70% ethanol (adult worms were separated from juveniles) and stored at  $-20^{\circ}\text{C}$ .

### DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

The DNA extractions were done on single nematodes or trematodes using a modified CTAB extraction method (Goodacre &

**Table 1.** Collection sites surveyed across the city of Nottingham and surrounding areas.

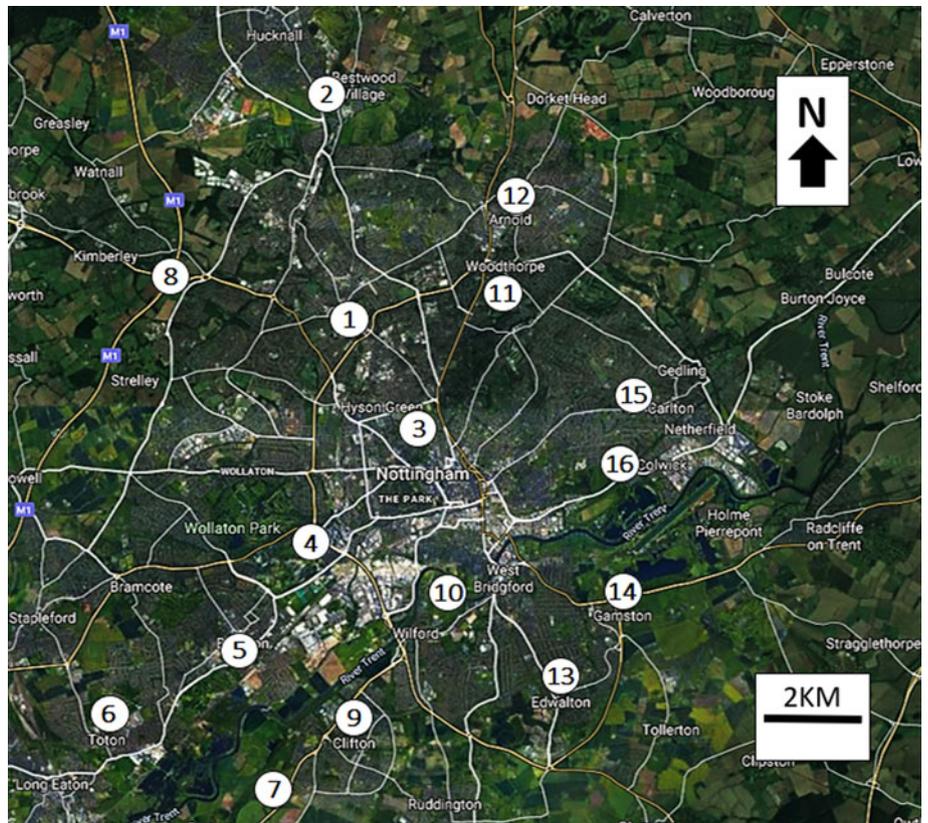
	Collection site	Code	Search area (km <sup>2</sup> )	Coordinates
1	Basford (Nottingham)	BAS	15,288	52.977957, -1.180909
2	Bestwood Country Park (Nottinghamshire)	MILL	116,987	53.025337, -1.184712
3	Forest Fields (Nottingham)	FOR	5132	52.96401, -1.159410
4	University Park Campus (Nottingham)	UNI	20,506	52.938199, -1.12508
5	Beeston (Nottinghamshire)	BEE	1583	52.922972, -1.214944
6	Toton (Nottinghamshire)	TOT	6469	52.915726, -1.264259
7	Attenborough Nature Reserve (Nottinghamshire)	ATEN	33,371	52.909117, -1.221000
8	Kimberley (Nottinghamshire)	KIM	5095	52.997686, -1.268583
9	Clifton South (Nottingham)	C-SOU	11,135	52.899179, -1.185660
10	Iremongers Pond (Nottingham)	POND	17,958	52.936184, -1.152757
11	Woodthorpe Grange Park (Nottingham)	GRAN	143, 670	52.982888, -1.135721
12	Arnot Hill Park (Nottingham)	ARNOT	45,220	52.997488, -1.133526
13	Edwalton (Nottinghamshire)	EDW	8181	52.917332, -1.124678
14	Gamston (Nottinghamshire)	GAM	24,538	52.928595, -1.108470
15	Carlton (Nottinghamshire)	CARL	37,525	52.965511, -1.103516
16	Colwick (Nottinghamshire)	COLW	15,920	52.952945, -1.091540

Wade, 2001). Extracted samples were resuspended in 100 µl of 10 mM (TRIS-HCl, pH 8.0) buffer. A list of extracted and genotyped samples for each site can be found in online supplementary tables 1 and 2. Promega GoTaq® G2 Master Mix buffer was used for all PCR reactions: 1 µl of DNA template was added to 24 µl of 1× Master Mix buffer (1U TAQ, 0.2 µM primers, 200 µM each dNTP, 1.5 mM MgCl<sub>2</sub>). The nematode DNA samples were identified using the region of the ribosomal RNA spanning the 18S-ITS1-5.8S-ITS2, which was amplified using the universal nematode primer set developed by Nadler *et al.* (2000) (N93: 5'-TTG AAC CGG GTA AAA GTC G-3' and N94: 5'-TTA GTT TCT TTT CCT CCG CT-3'). The trematode DNA samples were identified using the 18S rRNA gene, which was amplified using the universal trematode primer set developed by Kim *et al.* (2019) (LPF: 5'-AGG GAA TGG GTG GAT TTA TT-3' and LPR: 5'-AGA CAC GAC TGA AAG GTT GC-3'). The PCR conditions used were an initial 2 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 50°C and 2 min at 72°C, and finally 10 min at 72°C. PCR products were run and visualized on an ethidium bromide infused 1.5% agarose gel. PCR products were purified and sequenced using Macrogen's Eco-Seq service. Problematic sequences were re-amplified and sequenced using a

higher annealing temperature of 60°C to try to eliminate fungal contaminants amplifying instead of the parasite DNA.

### Parasite identification

Parasite sequences were first grouped together based on similarity, with sequences that were 99% identical being placed together. Next, the United States National Center for Biotechnology Information 'MOLE-BLAST Neighbor Search Tool' was used to find the closest matching reference sequences on the GenBank database (Altschul *et al.*, 1990; Benson *et al.*, 2013). This tool creates an alignment and a neighbour-joining tree to show the relationship that the query sequence has to the reference sequences in the GenBank non-redundant proteins database. Next, a secondary analysis was performed by placing our sequences within an alignment with all of the relevant closest matching GenBank reference sequences. This allowed us to create a maximum likelihood (ML) tree to see relationships between our sequences and the references taken from GenBank. The sequences were aligned in Seaview v5.0.5 (Gouy *et al.*, 2021) using the Muscle algorithm, with conserved sites being selected using the Gblocks program (Castresana, 2000). The phylogenetic trees were constructed



**Fig. 1.** Map of collection sites ( $n = 16$ ) across the city of Nottingham and surrounding areas (Google 2022).

using the ML method, using a general time reversible model incorporating gamma correction (GTR+ $\Gamma$ ) in PhyML v3.1 (Guindon *et al.*, 2010), with bootstrap analysis undertaken using 1000 replicates.

### GenBank accession numbers

The DNA sequences generated in this study are available in GenBank accession numbers OP626191 – OP626254 (online supplementary table 3).

## Results

### Infection rates

Of the 800 gastropods collected, 581 were slugs (Agriolimacidae, Arionidae, Boettgeriidae, Limacidae and Milacidae) and 219 were snails (Discidae, Helicidae, Hygromiidae and Oxylchilidae). The most common slug species found were *Deroceras invadens* (15%), *Tandonia budapestensis* (13%), *Deroceras reticulatum* (13%), *Arion hortensis* (10%), *Ambigolimax valentianus* (8%), *Limacus maculatus* (7%), *Arion vulgaris* (7%), *Tandonia sowerbyi* (6%), *Arion ater* (6%), *Arion subfuscus* (4%), *Arion rufus* (3%), *Arion silvaticus* (2%), *Limacus flavus* (2%), *Ambigolimax nyctelius* (1%), *Limax maximus* (1%), *Milax gagates* (<1%) and *Boettgerilla pallens* (<1%). The most common snail species found were *Cepaea nemoralis* (28%), *Cornu aspersum* (25%), *Cepaea hortensis* (20%), *Trochulus striolatus* (10%), *Oxychilus alliarius* (7%), *Monacha cantiana* (5%), *Discus rotundus* (3%), *Trochulus hispidus* (1%) and *Arianta arbustorum* (1%).

Overall, 227 specimens were infected (28%) with nematodes or trematodes (or both). Of those, 163 were slugs (28%) and 64 were

snails (29%) (table 2; fig. 2). The only gastropod species without any recorded infections were *A. arbustorum*, *B. pallens*, *D. rotundus* and *T. hispidus*. Nematodes were found in all other gastropods, with *T. budapestensis*, *D. invadens*, *C. aspersum*, *D. reticulatum*, *A. ater* and *C. nemoralis* accounting for over half of all infections. A total of 533 nematodes were recorded from 190 infected specimens (145 slugs and 45 snails). Of those, only 12 juvenile nematodes were found in 12 hosts (eight slugs and four snails) (table 2). Trematodes were rarer than nematodes, with *A. ater*, *A. hortensis*, *A. nyctelius*, *A. rufus*, *A. silvaticus*, *A. subfuscus*, *A. vulgaris*, *L. flavus*, *L. maximus* and *O. alliarius* having no recorded trematode infections. A total of 242 trematodes were recorded from 55 specimens (30 slugs and 25 snails) (table 2). Lastly, co-infections of both nematodes and trematodes were even rarer, with only 18 specimens being recorded as co-infected (13 slugs and five snails) (table 2).

Of the 16 sites surveyed, infection was found at all of them (table 3). The highest recorded rate of infection was 46% at site 7 (Attenborough Nature Reserve, Nottinghamshire) and site 13 (Edwalton, Nottinghamshire). The lowest recorded rate of infection was 12% at site 5 (Beeston, Nottinghamshire). Nematode infections were found at all 16 sites, with trematode infections found at 13 of the 16 sites (fig. 2). Specimens infected with both nematodes and trematodes were found at nine of the 16 sites.

### Nematode and trematode identifications

A total of 35 (23 adults, 12 juveniles) nematodes (online supplementary table 1) and 29 trematodes (online supplementary table 2) were genotyped. All sequences were grouped together based on similarity (>99%) and those groups were then matched with their closest GenBank references using the Basic Local Alignment Search Tool

**Table 2.** Gastropods collected and details of number of nematode and trematode (metacercariae) infections.

Family	Species	Number	Infected	Nematode	Trematode	Both
Agriolimacidae	<i>Deroceras invadens</i>	90	25	15	8	2
	<i>Deroceras reticulatum</i>	75	19	13	3	3
Arionidae	<i>Arion ater</i>	33	13	13	0	0
	<i>Arion hortensis</i>	59	11	11	0	0
	<i>Arion rufus</i>	20	5	5	0	0
	<i>Arion silvaticus</i>	14	2	2	0	0
	<i>Arion subfuscus</i>	25	6	6	0	0
	<i>Arion vulgaris</i>	42	8	8	0	0
	Boettgerillidae	<i>Boettgerilla pallens</i>	2	0	0	0
Discidae	<i>Discus rotundatus</i>	6	0	0	0	0
Helicidae	<i>Arianta arbustorum</i>	2	0	0	0	0
	<i>Cepaea hortensis</i>	44	7	6	1	0
	<i>Cepaea nemoralis</i>	62	14	9	4	1
	<i>Cornu aspersum</i>	54	24	14	7	3
Hygromiidae	<i>Trochulus hispidus</i>	3	0	0	0	0
	<i>Trochulus striolatus</i>	22	7	4	3	0
	<i>Monacha cantiana</i>	10	7	1	5	1
Limacidae	<i>Ambigolimax nyctelius</i>	5	1	1	0	0
	<i>Ambigolimax valentianus</i>	47	18	8	5	5
	<i>Limacus flavus</i>	10	3	3	0	0
	<i>Limacus maculatus</i>	42	9	8	0	1
	<i>Limax maximus</i>	3	2	2	0	0
Milacidae	<i>Milax gagates</i>	2	1	0	0	1
	<i>Tandonia budapestensis</i>	78	31	30	1	0
	<i>Tandonia sowerbyi</i>	34	8	7	0	1
Oxychilidae	<i>Oxychilus alliarius</i>	16	6	6	0	0
Total		800	227	172	37	18

Note: Gastropod species with zero infections are greyed out. 'Both' means a co-infection of nematodes and trematodes within a single specimen.

and MOLE-BLAST tool (ranked by lowest E-value). The nematode sequences fitted into seven groups, with all groups except group C2 having a GenBank reference match greater than 99% (table 4). The trematode sequences fitted into four groups, with all groups except group F1 having a GenBank reference match greater than 99% (table 4).

Next, ML trees were created for the nematode and trematode sequences by placing each group together with a range of related GenBank references. The majority of the groups were identified at the species level (fig. 3). Only groups C2 and F1 were not identifiable at the species level. Group C2 was outside of the *Cosmocerca/Cosmocercoides* genera (fig. 3C) and group F1 was outside of the *Opisthio glyphe/Macroderoides/Brachycoelium/Mesocoelium/Auridistomum/Telorchis* genera, respectively (fig. 3F).

## Discussion

### Rate of infection

The vast majority of gastropods collected and examined were slugs (73%), of which five families were represented

(Agriolimacidae, Arionidae, Boettgerillidae, Limacidae and Milacidae). The remaining gastropods were snails, of which four families were represented (Discidae, Helicidae, Hygromiidae and Oxychilidae). The largest families represented were the Arionidae (24%), Agriolimacidae (20%), Helicidae (20%), Milacidae (16%), Limacidae (13%), Hygromiidae (4%), Oxychilidae (2%), Discidae (<1%) and Boettgerillidae (<1%). The overall rate of infections for the gastropods collected was 28%. Both slugs (28%) and snails (29%) had a similar rate of infection. No lungworm species of medical or veterinary importance were found within the city of Nottingham. However, of the 26 gastropod species found, 16 are potential hosts for *An. vasorum*, eight are potential hosts for *Crenosoma vulpis* and five are potential hosts for *Ae. abstrusus* (online supplementary table 4).

### Nematodes

A total of 533 nematodes were isolated, with only 12 being juveniles. Juvenile nematodes are a useful indication for the



**Fig. 2.** Map of collection sites ( $n = 16$ ) across the city of Nottingham and surrounding areas showing infection rates at each collection site. White = uninfected, grey = nematode infection, dark grey = trematode infection and black = nematode/trematode co-infection (Google 2022).

**Table 3.** Infection rate of collected gastropods ( $n = 50$ ) at each site across the city of Nottingham and surrounding areas.

Collection site	Code	Infection rate	Nematode	Trematode
1 Basford (Nottingham)	BAS	40%	40%	8%
2 Bestwood Country Park (Nottinghamshire)	MILL	16%	8%	8%
3 Forest Fields (Nottingham)	FOR	28%	22%	8%
4 University Park Campus (Nottingham)	UNI	16%	10%	8%
5 Beeston (Nottinghamshire)	BEE	12%	12%	0%
6 Toton (Nottinghamshire)	TOT	20%	20%	0%
7 Attenborough Nature Reserve (Nottinghamshire)	ATEN	46%	46%	0%
8 Kimberley (Nottinghamshire)	KIM	36%	32%	8%
9 Clifton South (Nottingham)	C-SOU	28%	26%	2%
10 Iremongers Pond (Nottingham)	POND	14%	12%	4%
11 Woodthorpe Grange Park (Nottingham)	GRAN	22%	20%	2%
12 Arnot Hill Park (Nottingham)	ARNOT	26%	24%	2%
13 Edwalton (Nottinghamshire)	EDW	46%	42%	6%
14 Gamston (Nottinghamshire)	GAM	40%	20%	28%
15 Carlton (Nottinghamshire)	CARL	30%	24%	12%
16 Colwick (Nottinghamshire)	COLW	34%	30%	8%

**Table 4.** BLAST-MOLE results (ranked by E-value) for grouped nematode (groups A–D) and trematode (groups E–F) sequences with their top five closest references.

Group	Samples	Closest references	Reference name	% match
Nematodes				
A1	EDW 5 FOR 20 FOR 26 GRAN 1 GRAN 13 UNI 15	MK214813	<i>Agfa flexilis</i>	99.4
		FJ516760	<i>Phasmarhabditis neopapillosa</i>	87
		MF192968	<i>Angiostoma margaretae</i>	86
		FJ516761	<i>Phasmarhabditis hermaphrodita</i>	85
		MK214815	<i>Angiostoma gandavensis</i>	81
B1	ARNOT 1 ARNOT 11 ARNOT 35 (J) BAS 45 BEE 12 BEE 14 CARL 18 COLW 13 (J) C-SOU 1 C-SOU 7 C-SOU 9 EDW 1 (J) EDW 2 FOR 36 (J) GAM 1	MF192968	<i>Angiostoma margaretae</i>	99.4
		MK214816	<i>Angiostoma norvegicum</i>	92
		MK214815	<i>Angiostoma gandavensis</i>	87
		FJ516761	<i>P. hermaphrodita</i>	83
		FJ516760	<i>P. neopapillosa</i>	82
		B2	BEE 16 C-SOU 3 KIM 1 KIM 33	MK214815 MF192968 MK214816 FJ516761 FJ516760
C1	POND 14	OL472311	<i>Cosmocerca longicauda</i>	99.9
		LC018444	<i>Cosmocercoides pulcher</i>	90
		MH178312	<i>Cosmocercoides qingtianensis</i>	90
		AB908161	<i>Cosmocercoides tonkinensis</i>	90
		MN839761	<i>Cosmocerca simile</i>	96
C2	BAS 1 (J) BEE 1 (J) KIM 40 (J) MILL 19 (J)	OL472311	<i>C. longicauda</i>	90
		LC018444	<i>Co. pulcher</i>	88
		MH178312	<i>Co. qingtianensis</i>	88
		AB908161	<i>Co. tonkinensis</i>	88
		MN839761	<i>Co. simile</i>	96
D1	ATEN 12 (J) TOT 24 TOT 25 (J)	FJ516761	<i>P. hermaphrodita</i>	99.3
		FJ516760	<i>P. neopapillosa</i>	90
		MK214815	<i>A. gandavensis</i>	84
		MF192968	<i>A. margaretae</i>	79
		MK214813	<i>Ag. flexilis</i>	85
D2	C-SOU 10 (J) GAM 16 (J)	FJ516760	<i>P. neopapillosa</i>	99.2
		FJ516761	<i>P. hermaphrodita</i>	90
		MK214815	<i>A. gandavensis</i>	82
		MF192968	<i>A. margaretae</i>	78
		MK214813	<i>A. flexilis</i>	86

(Continued)

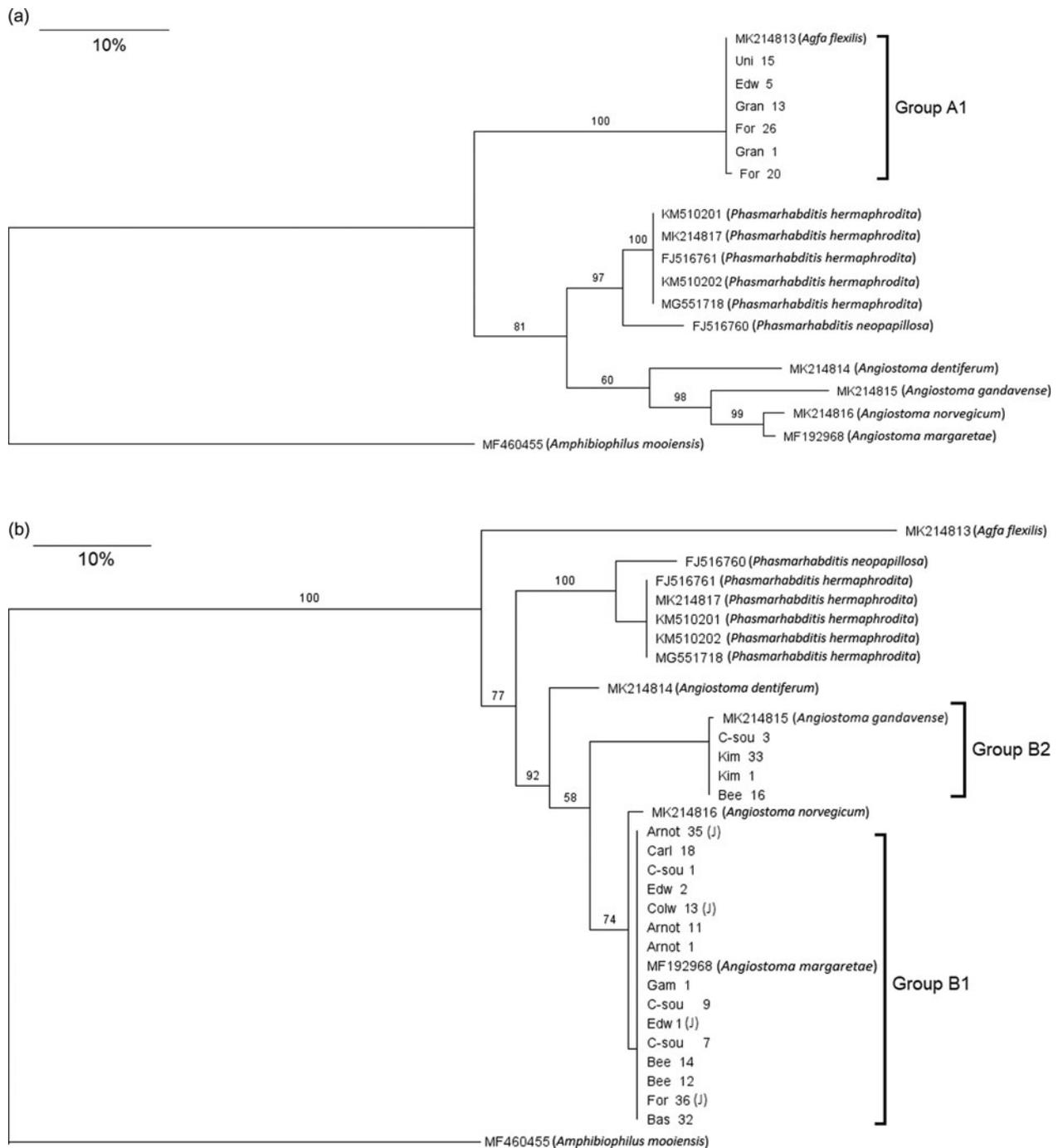
Table 4. (Continued.)

Group	Samples	Closest references	Reference name	% match
Trematodes				
E1	BAS 11	KT074950	<i>Brachylaima arcuata</i>	99.6%
	FOR 23			
	GRAN 8	KT074955	<i>Brachylaima mesostoma</i>	98%
	KIM 3			
	KIM 10			
	KIM 37			
	MILL 4a	KT074952	<i>Brachylaima fuscata</i>	97%
	MILL 4b			
	MILL 31	AY222085	<i>Brachylaima thompsoni</i>	97%
	MILL 32			
MILL 35				
POND 5				
POND 8				
UNI 5	KP903630	<i>Urotocus rossitensis</i>	94%	
E2	ARNOT 18	KT074952	<i>B. fuscata</i>	99.8
	BAS 26			
	COLW 2	AY222085	<i>B. thompsoni</i>	99.4
	EDW 8			
	EDW 25	KT074955	<i>B. mesostoma</i>	99.2
	FOR 4			
	GAM 3			
	GAM 15	KT074950	<i>B. arcuata</i>	98
	GAM 16			
	GAM 26	KP903638	<i>Michajlovia migrata</i>	96
KIM 40				
E3	CARL 12	KT074955	<i>B. mesostoma</i>	100
	CARL 13	AY222085	<i>B. thompsoni</i>	99.6
	C-SOU 19	KT074952	<i>B. fuscata</i>	99.2
		KT074950	<i>B. arcuata</i>	99
		KP903638	<i>M. migrata</i>	96
F1	UNI 39	AY222156	<i>Telorchis assula</i>	97
		AY222160	<i>Brachycoelium salamandrae</i>	96
		AY222159	<i>Auridistomum chelydrae</i>	96
		JQ886404	<i>Mesocoelium lanfrediae</i>	96
		MZ787582	<i>Opisthioglyphe ranae</i>	96

Note: (J) indicates it was a juvenile nematode. Each of the different designated groupings of ITS (nematode) and 18S (trematode) sequences are less than 1% different. Nematode and trematode groups with less than 99% GenBank reference match are coloured grey.

possible presence of lungworm (metastrongyloid) species of veterinary importance such as *An. vasorum*. Of those 12 juvenile nematodes, no lungworm species were found. Instead, four of them were identified as *Angiostoma margaretae* (Angiostomatidae), a parasite whose definitive host has been reported to be a milacid slug species (Ross *et al.*, 2017). We also found it inside *D. invadens* (Agriolimacidae) and *A. valentianus* (Limacidae). Four were identified as an unknown Cosmocercidae species, a family of parasitic nematodes whose definitive hosts are reptiles and amphibians (Baker, 1984). Two were identified as *Phasmarhabditis hermaphrodita* and two were identified as *Phasmarhabditis neopapillosa* (Rhabditidae). *Phasmarhabditis* is a genus of facultative parasitic nematodes that can parasitize a broad range of gastropod species (Andrus & Rae, 2019). Of the adult nematodes identified, all were of non-medical (or veterinary) relevance, belonging to four of the seven gastropod-related nematode families (Aegidae, Angiostomatidae, Cosmocercidae and Rhabditidae).

The interactions these nematode families have with terrestrial gastropods are poorly understood (Wilson & Grewal, 2005). The most understood species is *P. hermaphrodita*, which has been developed into an effective biological alternative molluscicide (Nemaslug®) that reduces agricultural damage done by gastropod pests (Rae *et al.*, 2007). Unlike chemical molluscicides, Nemaslug® has no adverse effects on non-target organisms such as beneficial organisms (acarids, annelids, carabids, collembolans, dipterans, isopods and nematodes), or gastropod predators (amphibians, birds, mammals and reptiles) (Iglesias *et al.*, 2003). However, unlike chemical molluscicides, Nemaslug® cannot kill every gastropod pest species. This is due to *P. hermaphrodita* only being able to kill smaller gastropod species (e.g. *Deroceas* spp. and *Arion hortensis*) and the juveniles of some larger species (*Arion ater* and *Cornu aspersum*) (Rae, 2017), while larger gastropod species (*Ambigolimax* spp., *Cepaea hortensis*, *Limacus* spp., *Limax* spp. and *Lissachatina fulica*) are resistant to the fatal effects of *P. hermaphrodita* (Williams & Rae, 2015; Rae, 2017).



**Fig. 3.** Maximum likelihood phylogenetic trees of different nematode (trees A–D) and trematode (trees E–F) species using the ITS and 18S rRNA gene, respectively. Tree A was created using 325 base pairs (bp) of the ITS and is rooted on *Amphibiophilus mooiensis*. Tree B was created using 306 bp of the ITS and is rooted on *A. mooiensis*. Tree C was created using 402 bp of the ITS and is rooted on *Paraspidodera uncinata*. Tree D was created using 409 bp of the ITS and is rooted on *A. mooiensis*. Tree E was created using 450 bp of the 18S rRNA and is rooted on *Michajlovina turdi*. Tree F was created using 456 bp of the 18S rRNA and is rooted on *Brachycladium goliath*. All trees were generated using PhyML v3.1; the numbers on the branches indicate the bootstrap percentages for 1000 replicates (bootstrap values under 50% are not shown). The scale bar represents percentage sequence divergence. Differing alignment lengths are due to the limited length of GenBank references. Accession numbers for all sequences can be found in online supplementary table 3.

### Trematodes

A total of 242 trematodes were isolated. Of these, 29 were genotyped, 14 were identified as *Brachylaima arcuata*, 11 were identified as *B. fuscata* and three were identified as *B. mesostoma*. All these *Brachylaima* species are common gastrointestinal parasites

of the bird families Corvidae, Sylviidae and Turdidae (Heneberg *et al.*, 2016). One other trematode sample (belonging to group F1) could not be identified at the species-level. It clustered closely with the genera *Opisthioglyphe*, *Macroderoides*, *Brachycoelium*, *Mesocoelium*, *Auridistomum* and *Telorchis*, placing it within the

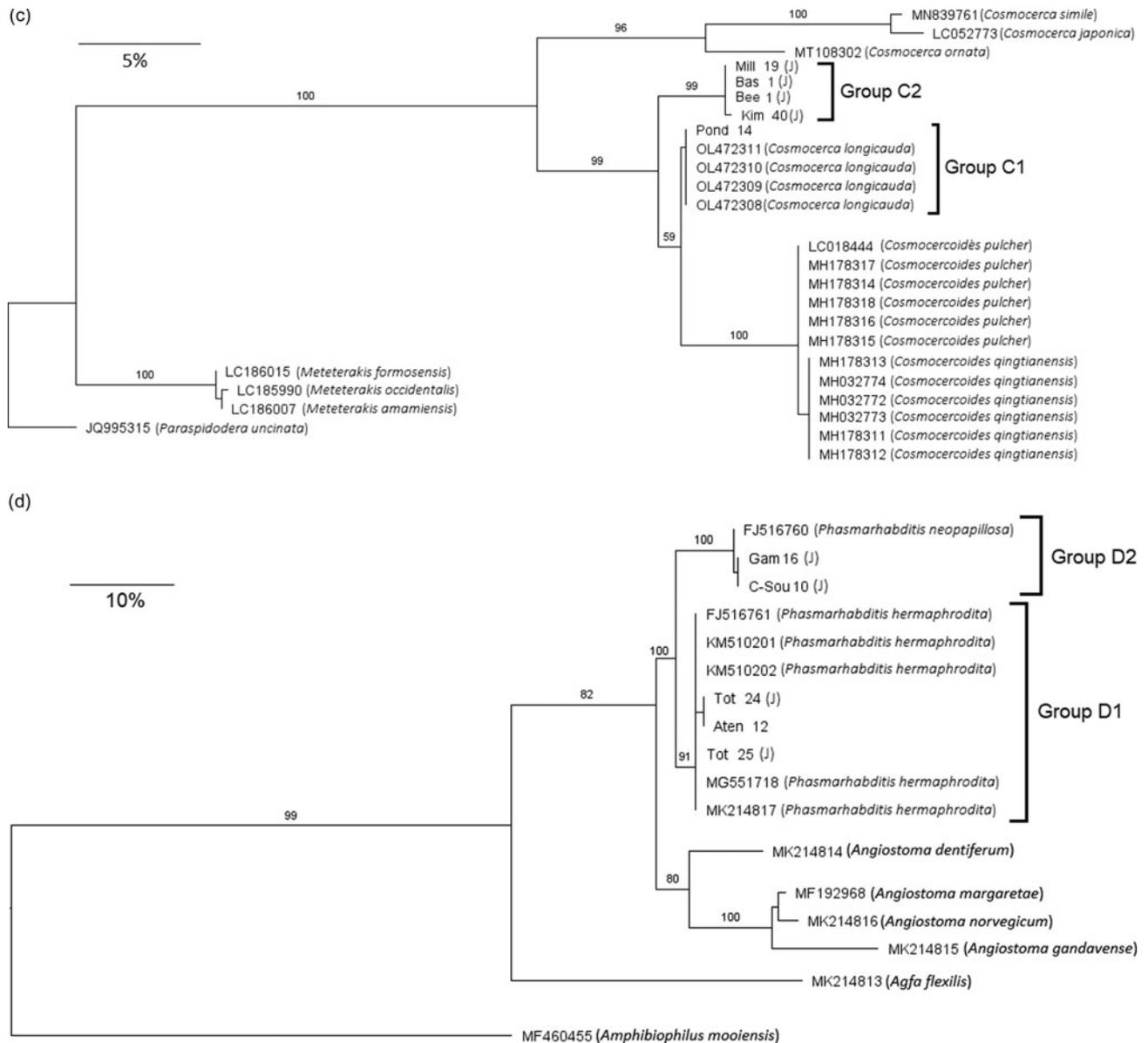


Fig. 3. Continued.

Plagiorchioidea superfamily. Genera of this Plagiorchioidea superfamily are common parasites of amphibians, fishes and reptiles (Tkach *et al.*, 2001).

*Brachylaima* is a common gastrointestinal parasite of birds, mammals, and reptiles. There are over 60 described species, with *Brachylaima* being found in Africa, the Americas, Asia, Europe, and Oceania (Nasir & Rodriguez, 1966; Wheeler *et al.*, 1989; Richards *et al.*, 1995; Awharitoma *et al.*, 2003; Butcher & Grove, 2005; Richardson & Campo, 2005; Gállego *et al.*, 2014; Gracenea & Gállego, 2017; Nakao *et al.*, 2017; Gérard *et al.*, 2020; Termizi & Him, 2021). *Brachylaima cribbi* is the only documented species capable of infecting humans (Butcher & Grove, 2001) with brachylaimiasis first documented in 1996, with 13 more cases in the subsequent decades after its discovery, all occurring in Australia (Butcher *et al.*, 1996; Gállego & Gracenea, 2015). Brachylaimiasis causes diarrhoea, abdominal pain, anorexia, eosinophilia and weight loss (or decreased weight gain) in infected humans, with a predicted mortality rate of 5–10% in untreated patients

(Gállego & Gracenea, 2015). Transmission is typically from either the consumption of undercooked land snails (such as *Cornu aspersum*) infected with metacercariae, or the unintentional consumption of infected gastropod slime/faeces/corpse-contaminated fruits and vegetables (Butcher & Grove, 2001).

While the consumption of snails is unpopular in the United Kingdom, on average the world consumes 450,000 tonnes (496,040 US tons) of edible snails every year, of which only 15% come from snail farms (López *et al.*, 2015). Spain, France, Portugal and Belgium are the biggest importers of snails, with approximately 17 million kilograms of snails being imported as a whole from 2020–2021 (United Nations, 2022). Concerns about the rates of *Brachylaima* infection in *C. aspersum* at farms and markets has already been raised in France and Spain (Gállego & Gracenea, 2015; Gracenea & Gállego, 2017; Gérard *et al.*, 2020). It is unknown what effect non-*B. cribbi* species have on public health as there are no studies exploring the possibility of brachylaimiasis caused by European *Brachylaima* species. Furthermore, brachylaimiasis

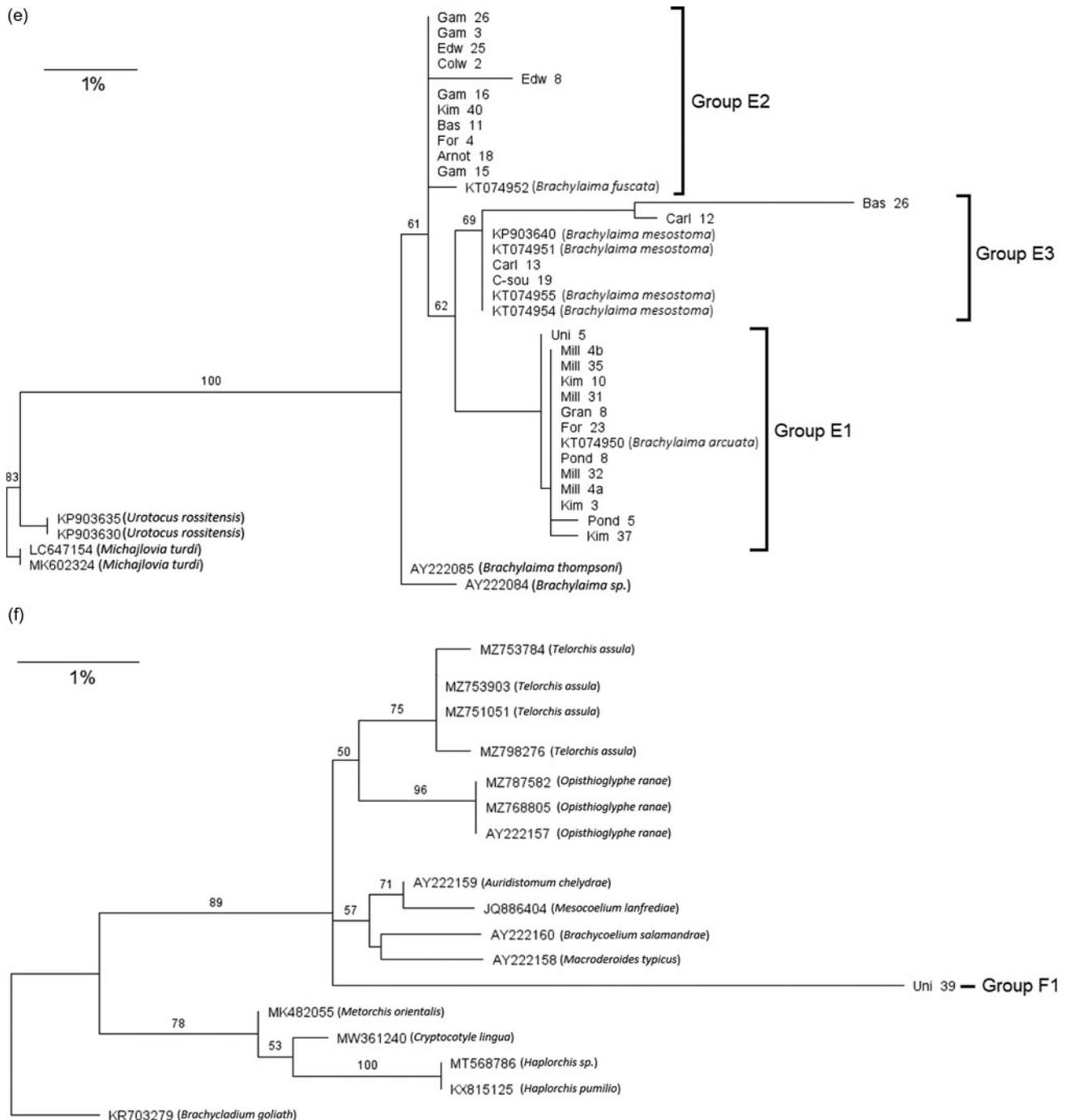


Fig. 3. Continued.

could be frequently misdiagnosed or overlooked in Europe due to either a lack of experience in identifying it or due to how small *Brachylaima* eggs are in human faeces (<30 µm in length) (Gracenea & Gállego, 2017).

**Supplementary material.** To view supplementary material for this article, please visit <https://doi.org/10.1017/S0022149X22000645>.

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