Parasitic nematodes of the genus *Syphacia* Seurat, 1916 infecting Muridae in the British Isles, and the peculiar case of *Syphacia frederici*

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SUMMARY

Syphacia stroma (von Linstow, 1884) Morgan, 1932 and *Syphacia frederici* Roman, 1945 are oxyurid nematodes that parasitize two murid rodents, *Apodemus sylvaticus* and *Apodemus flavicollis,* on the European mainland. Only *S. stroma* has been recorded previously in *Apodemus* spp. from the British Isles. Despite the paucity of earlier reports, we identified *S. frederici* in four disparate British sites, two in Nottinghamshire, one each in Berkshire and Anglesey, Wales. Identification was based on their site in the host (caecum and not small intestine), on key morphological criteria that differentiate this species from *S. stroma* (in particular the tail of female worms) and by sequencing two genetic loci (cytochrome C oxidase 1 gene and a section of ribosomal DNA). Sequences derived from both genetic loci of putative British *S. frederici* isolates formed a tight clade with sequences from continental worms known to be *S. frederici*, clearly distinguishing these isolates from *S. stroma* which formed a tight clade of its own, distinct from clades representative of *Syphacia obvelata* from *Mus* and *S. muris* from *Rattus*. The data in this paper therefore constitute the first record of *S. frederici* from British wood mice, and confirm the status of this species as distinct from both *S. obvelata* and *S. stroma*.

Key words: *Syphacia frederici*, *Syphacia stroma*, *Syphacia obvelata*, *Syphacia muris*, oxyurid nematodes, *Apodemus sylvaticus*, *Apodemus flavicollis*.

INTRODUCTION

The nematode genus *Syphacia* Seurat, 1916 (Oxyuridae Cobbold, 1864: Syphaciinae Railliet, 1916) members of which are colloquially often referred to as pinworms (Adamson, 1994; Grear and Hudson, 2011), is a cosmopolitan genus parasitizing rodents. Hugot (1988) separated the genus into three subgenera including *Syphacia* (*Syphacia*) comprising 14 species, all occurring in Cricetidae Fisher, 1817 or Muridae Illiger, 1811. Since that time more than 25 new species have been described in this subgenus, particularly from Southeast Asia, Indonesia, South America and Australia (see, e.g. Hasegawa and Tarore, 1996; Weaver and Smales, 2010; Rojas *et al*. 2011, Smales, 2011; Dewi *et al*. 2014) such that the subgenus now encompasses more than 39 species. It is generally assumed that species of *Syphacia* are narrowly host specific, partly because the French school considered them *a priori* to have co-evolved with their host rodents (Hugot, 1988). *Syphacia* species may have exacting dietary requirements which restrict them to one or a few congeneric hosts, although under intensive mixed husbandry of rodents, cross-infections can occur, for example, the occurrence of *S. obvelata* (Rudolphi, 1802) in laboratory rats and *S. muris* (Yamaguti, 1935) in laboratory mice (Hussey, 1957). It has also been possible to experimentally infect hosts that do not normally harbour particular species in the wild (*Syphacia stroma* in laboratory mice, Lewis, 1968). Nevertheless, while most rodents in nature harbour only their own specific species of *Syphacia*, casual infection by more generalist species (ecological fitting) may also have occurred (Araujo *et al*. 2015; Weaver *et al*. 2016).

Species of *Syphacia* are perhaps best known as unwanted contaminants of laboratory rodents

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(Taffs, 1976) that have been bred for medical research, under husbandry conditions specifically designed to eliminate entirely, or at the very least to minimize the possibility of infection with specific pathogens (referred to as specific pathogen-free mice and rats). Control of these nematodes is made difficult as a result of their extremely efficient transmission strategies; for example, egg laying on the perianal surface of their hosts during sleep, very short life cycles including rapid embryonation of eggs and rapid maturation in the host, and eggs that are resilient but sticky and capable of adhering to host hair, dust and materials in the bedding area (see references in Lewis, 1968; Lewis and D'Silva, 1986; Kerboeuf and Lewis, 1987; Lewis, 1987; Adamson, 1994; Grear and Hudson, 2011; Meade and Watson, 2014).

In the Euro-Siberian region of the Palearctic, wood mice (*Apodemus sylvaticus*) and yellow-necked mice (*Apodemus flavicollis*) are parasitized by two *Syphacia* species. Perhaps the best known of these is *S. stroma* (von Linstow, 1884) Morgan, 1932, which has been reported throughout the range of *Apodemus* spp. in Europe [e.g. Ireland (Loxton *et al*. 2016), Portugal (Eira *et al*. 2006) and Fauna Europaea (http://www.fauna-eu.org) lists 16 countries for this species]. *Syphacia stroma* is an unusual and atypical member of the genus, because in contrast to all other known members of the genus, this species lives mainly in the small intestine of its host, although migrating patent females may be found in the caecum and colon, and in heavy infections there can be a spill over of worms from the small into the large intestine. All other known species of *Syphacia* are parasites of the large intestine, living predominantly in the caecum of their rodent hosts but also in the colon, environments that are rich in the bacteria upon which these nematodes feed. In our experience, and that of many other workers, infections with *S. stroma* in wood mice can be huge, exceeding many hundreds and even thousands of worms per host, and prevalence has generally been recorded as high, so clearly this is one of the dominant parasitic nematodes infecting wood mice in the region (Thomas, 1953; Behnke *et al*. 1999; Abu-Madi *et al*. 2000).

The second species of *Syphacia* infecting *Apodemus* spp. in Europe is *S. frederici* Roman, 1945, which is a more recently recognized caecal dwelling species that has been widely recorded throughout the range of its hosts in Europe [e.g. in former Czechoslovakia (Tenora *et al*. 1974); Italy (Milazzo *et al*. 2010); Portugal (Eira *et al*. 2006); Romania (Mészáros and Murai, 1979); Serbia (Čabrilo *et al*. 2016) and 10 countries listed in Fauna Europaea (http://www.fauna-eu.org)], but to our knowledge has apparently never been reported in wood mice from the British Isles [personal Communication; Eileen Harris and records at the Natural History Museum in London (NHM)]. Arguably, the rodent–helminth fauna of the British Isles ranks among the better studied rodent–helminth compound communities in the world (Elton *et al*. 1931; Thomas, 1953; Sharpe, 1964; Lewis, 1968; Canning *et al*. 1973; Murúa, 1978; Montgomery and Montgomery, 1990; Abu-Madi *et al*. 1998; 2000), and it seems to have been well accepted by workers in this field that the only *Syphacia* endemic in wood mice in the British Isles is *S. stroma* (Lewis, 1987).

However, while conducting surveys of helminth communities in the last decade in various sites in the British Isles, we have encountered wood mice harbouring *Syphacia* infections restricted to the caecum and colon of their hosts and suspected that these might be *S. frederici*. Intrigued by the lack of any earlier reports of *S. frederici* in the UK, we conducted a thorough investigation of the suspected worms. In this paper we compare their morphology to published reports of *S. frederici*, and provide novel data on their genetic signature, showing how they relate to other common *Syphacia* species parasitizing murid rodents in the British Isles. For comparison we draw on additional *Syphacia* spp. worms recovered from murid hosts from some other locations in Europe. We also emphasize the key morphological features that can be used easily in quantitative studies to distinguish between *S. stroma* and *S. frederici*.

MATERIALS AND METHODS

Sources of worms

The sources of worms used for genetic analysis are shown in Table 1, and the approximate locations are illustrated on a map of Europe in Fig. 1. Rodents were trapped using Longworth, Sherman and other humane live capture traps. Traps were set at dusk and collected soon after dawn. Animals were inspected, culled by cervical dislocation and the entire intestinal tract was preserved in 80% ethanol, for subsequent dissection in our laboratory. In the laboratory, the intestinal tracts were divided into stomach, small intestine (three sections corresponding approximately to duodenum, jejunum and ilium), caecum and colon, and the contents of each section were examined carefully in separate Petri dishes ensuring no contamination with material from other sections. All recovered worms were transferred to 80% ethanol in 1·5 mL tubes and frozen at −80 °C until further processing for DNA extraction. All tubes were given a unique identifier reference (Table 1) and a range of pertinent details of each host was recorded on our database.

Some worms were obtained from our collaborators and the details of processing varied. In Ireland, mice were first euthanized with an overdose

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Table 1. Details of the worms that were sequenced (CO1 and/or rDNA) for the current study and/or examined morphologically

Reference	Host	Source	Putative species
BERKSHIRE-17As01Sf[a]	*Apodemus sylvaticus*	West Berkshire, England	*Syphacia frederici*
NOTTINGHAM-13As01Sff[a]	*A. sylvaticus*	Nottingham site 1, England	*S. frederici*
NOTTINGHAM-12As74Sff[a]	*A. sylvaticus*	Nottingham site 1, England	*S. frederici*
NOTTINGHAM-AL J16·1	*A. sylvaticus*	Nottingham site 2, England	*S. frederici*
POLAND-16As-DD-24Sf[a]	*A. sylvaticus*	Mazury, Poland	*S. frederici*
POLAND-GZA102-MSf[b]	*Apodemus flavicollis*	Wrocław, Poland	*S. frederici*
PORTUGAL-13As18Sf[a]	*A. sylvaticus*	Pancas, Portugal	*S. frederici*
PORTUGAL-13As21Sf[b]	*A. sylvaticus*	Pancas, Portugal	*S. frederici*
WALES-GWYN-15As01Sff[a]	*A. sylvaticus*	Anglesey, Wales	*S. frederici*
NOTTINGHAM-13Md01Sof	*Mus domesticus*	Nottingham, site 3, England	*Syphacia obvelata*
POLAND-Balb/c PHMmSo1	*Mus musculus* (BALB/c)	Warsaw University, Poland	*S. obvelata*
POLAND-GZMmSo	*M. musculus* (BALB/c)	Wrocław University, Poland	*S. obvelata*
SCOTLAND-IOM-15Mm352S	*M. domesticus*	Isle of May, Scotland	*S. obvelata*
SCOTLAND-IOM-15Mm358S	*M. domesticus*	Isle of May, Scotland	*S. obvelata*
DORSET- 11As24Ss	*A. sylvaticus*	Dorset, England	*Syphacia stroma*
DURHAM-06As01Ss	*A. sylvaticus*	Durham, England	*S. stroma*
EIRE-16As-PS-M134Ss	*A. sylvaticus*	Limerick, Ireland	*S. stroma*
FRANCE-12Apo11Ss	*A. sylvaticus*	Brittany, France	*S. stroma*
JERSEY-14As01Ssf2	*A. sylvaticus*	Le Braye, Jersey	*S. stroma*
NORFOLK-12As60Ss	*A. sylvaticus*	Norfolk, England	*S. stroma*
POLAND-GZAf48-8Ss[a]	*A. flavicollis*	Wrocław, Poland	*S. stroma*
PORTUGAL-12Apo3Ss[b]	*A. sylvaticus*	Pancas, Portugal	*S. stroma*
PORTUGAL-13As16Ss[a]	*A. sylvaticus*	Pancas, Portugal	*S. stroma*
SCOTLAND-06As52Ss	*A. sylvaticus*	Edinburgh, Scotland	*S. stroma*
WALES-GWYN-12As23Ss	*A. sylvaticus*	Anglesey, Wales	*S. stroma*
NETHERLANDS-15RnFF1512/25Sm	*Rattus norvegicus*	Friesland, The Netherlands	*Syphacia muris*

All specimens of *S. stroma* were recovered from the small intestine of the wood mice. All *S. frederici* and *S. obvelata* were recovered from the caecum of wood mice and *S.muris* from the caecum of rats.
The reference column gives the identity of the animal in our database and frozen helminth library, but for this study we only provide the genetic sequence of one worm extracted from each of these hosts.
Nottingham site 1 = suburban garden in West Bridgford, Nottingham.
Nottingham site 2 = suburban garden in Beeston, Nottingham.
Nottingham site 3 = Farm in Tollerton, Nottingham.
[a] Specimens also examined morphologically.
[b] Only examined morphologically.

of isoflurane, following which their digestive tracts were immediately removed and frozen at −20 °C for subsequent dissection and recovery of worms. In the Netherlands, rats were anaesthetized with isoflurane and were euthanized by cardiac puncture. Upon death, the intestinal tract including the stomach was taken from each rat for parasitological examination and was stored either at +4 °C for immediate evaluation or at −20 °C until further use (Franssen *et al*. 2016).

Molecular genetic comparison of species

DNA was isolated from individual worms using Extracta™ DNA prep kits (Quantabio) or DirectPCR lysis buffer (Viagen Biotech) according to the manufacturer's instructions. The cytochrome C oxidase 1 (CO1) gene was amplified using primers (forward: 5′-TGGTCTGGTTTTGTTGGTAG TT-3′, reverse: 5′-AACCACCCAACGTAAACAT AAA-3′; Okamoto *et al*. 2007). The ribosomal DNA (rDNA) region consisting of internal transcribed spacer (ITS)-1, 5·8S gene and ITS-2 (~700–750 bp) was amplified in separate reactions using the universal NC5 forward (5′-GTAGGTGAACCT GCGGAAGGATCATT-3′) and NC2 reverse primers (5′-TTAGTTTCTTTTCCTCCGCT-3′; Newton *et al*. 1998, Table 2). PCR reactions contained: 12·5 μL of BioMix Red (Bioline), 0·5 μM of the forward and reverse primers, <250 ng of template DNA and nuclease-free water to a total volume of 25 μL. AccuStart II Taq Polymerase (Quantabio) was used in place of BioMix Red to improve amplification of the CO1 gene, according to the manufacturer's instructions. Thermal cycling conditions for CO1 were: denaturation for 3 min at 94 °C, then 35 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min 30 s at 72 °C, with a final extension time of 7 min at 72 °C before being held at 4 °C. Thermal cycling conditions for the rDNA region were: denaturation for 1 min at 94 °C, then 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C, with a final extension of 72 °C for 5 min before being held at 4 °C. All PCR reactions were conducted in a Biorad PCT-200 thermocycler.

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Fig. 1. The location of rodents that were sampled in the current study. 1 = Nottingham, England; 2 = Anglesey, Wales; 3 = Mazury, Poland; 4 = Pancas, Portugal; 5 = Warsaw, Poland; 6 = Wrocław, Poland; 7 = Limerick, Ireland; 8 = Brittany, France; 9 = Jersey, British Isles; 10 = Norfolk, England; 11 = Edinburgh, Scotland; 12 = Friesland, Netherlands; 13 = Isle of May, Scotland; 14 = Berkshire, England; 15 = Dorset, England; 16 = Durham, England.

Table 2. Sequences used in the current study taken from GenBank

Gene	Host	Syphacia	Location and specimen code	Accession
CO1	*Mus musculus*	*Syphacia obvelata*	Japan-Tottori-SobTRMM	AB282591
CO1	*Mus caroli*	*Syphacia ohaorum*	Japan-Okinawa-SohOKMC	AB282592
CO1	*Apodemus speciosus*	*Syphacia frederici*	Japan-Okayama-SfrHZAP	AB282586
CO1	*A. speciosus*	*S. frederici*	Japan-Oita-SfrOTAS	AB282593
CO1	*A. speciosus*	*S. frederici*	Japan-Hokkaido-SfrHDAS	AB282588
CO1	*A. speciosus*	*S. frederici*	Japan-Iwae-SfrMOAP	AB282587
CO1	*Apodemus argenteus*	*Syphacia emileromani*	Japan-Ehime-SemSJAA	AB282590
CO1	*A. speciosus*	*Syphacia agraria*	Japan-Hokkaido-SagHDAS	AB282589
rDNA	*M. musculus*	*S. obvelata*	Taiwan-Lab-EU263105·2	EU263105
rDNA	*Rattus norvegicus*	*Syphacia muris*	Taiwan-Lab-EU263106·2	EU263106

Amplification in all PCR reactions was confirmed by visualization on a 1× SYBRSafe™, 1·5% agarose gel. PCR products were purified using ExoSAP (Affymetrix) and the final DNA concentration estimated by Nanodrop before dilution with nuclease-free water to the required concentration for sequencing. Sequencing primers, identical to the amplification primers, were diluted to the required concentration with nuclease-free water and supplied to Source Bioscience, along with PCR products, for Sanger sequencing. Chromatograms were inspected visually for ambiguities.

Sequence alignments were produced within the Mega 6·0 package using ClustalX followed by visual inspection. Phylogenetic analysis was performed with RAxML (v8·2·9) *via* the CIPRES Science Gateway using the maximum likelihood algorithm. Analysis of the rDNA ITS and 5·8S regions was carried out using the full sequence including indels. The *Syphacia* spp. DNA sequences acquired from *Apodemus* spp. and *Mus* spp. by Okamoto *et al*. (2007) were included in the CO1 alignments (Table 2). The *S. obvelata* and *S. muris* sequences produced by Parel *et al*. (2008)

were included in the rDNA alignment. Voucher sequences, including all sequences generated by this study and included in the current paper, have been deposited in GenBank (CO1 – MF142419 to MF142433; rDNA – MF142434 to MF142456).

Methods utilized for morphological comparison of species

Individual worms, voucher specimens taken from the samples of worms used for genetic analysis, were cleared in lactophenol and examined microscopically as temporary wet mounts. Measurements in micrometres, unless otherwise stated, were taken using an eyepiece micrometre and light micrographs using an Olympus photomicrographic system. Specimens of *S. stroma* from the NHM registration numbers 1934·7·19. 10–15; 1956·8·16·3–6; 1970·55–62. 63–68; 1979. 175–194; 983·3928–3929 were also examined. Specimens of *S. stroma* from Portugal (SAM AHC 47961 and 47962), and *S. frederici* from Portugal (SAM AHC 47963 and 47964), from Poland (SAM AHC 47965), from Wales (SAM AHC 47966) and from Nottingham (SAM AHC 47967) have been deposited in the South Australian Museum, Adelaide, with registration numbers shown in parenthesis. Additional specimens were deposited in the NHM with voucher numbers as follows: NORFOLK 12As60Ss, *S. stroma*, NHMUK 2017·5·19·1–6; WALES-GWYN-12As23Ss, *S. stroma*, NHMUK 2017·5·19·7–12; NOTTINGHAM-12As74Sff, *S. frederici*, NHMUK. 2017·5·19·13–18; WALES-GWYN-15As01Sff, *S. frederici*, NHMUK.2017·5·19·19–24.

RESULTS

Molecular genetic comparison of worms

The rDNA primers amplified 709–796 bps of DNA with a large number of nucleotide insertion/deletion events (indels) giving rise to the differences in length between clades. To the best of our knowledge, there are no published sequences for this target region in either *S. stroma* or *S. frederici*. As a result of the indels the *S. stroma* clade was uniformly 753 bp while *S. frederici* was 710 bp, the result of 69 nucleotide indels between the two species. Within clades, the nucleotide sequence was highly uniform with the *S. stroma* clade containing only two single nucleotide polymorphisms (SNPs) and *S. frederici* containing only one.

For *S. obvelata* and *S. muris*, we were able to compare our rDNA sequences to published data from GenBank. For *S. obvelata* there were three SNPs in the ITS-1 region; one [a site 28 bp (site 28) from the start of the primer binding region: T/G] in the Scotland (IOM-16Mm352S, IOM-16Mm358S) and Nottingham (13Md013Sf) isolates, another in the Scotland isolates only (site 62: T/A) and a third in the Nottingham isolate only (site 240: G/T). A fourth SNP occurred in the Nottingham (13Md01Sf) ITS-2 region (site 618: C/A). Therefore, the sequence for *S. obvelata* whether from laboratory mice from Poland or from wild caught mice in the UK was very similar to the published sequence for this species; although interestingly British isolates appear to have diverged slightly from European and laboratory isolates. Likewise our sequence for *S. muris* from wild caught brown rats from the Netherlands was 100% identical across a 796 bp region to laboratory worms from Taiwan published in GenBank (Table 2 and Parel *et al*. 2008).

Our genetic data unambiguously confirm that *S. stroma* and *S. frederici* are indeed two distinct species and that molecular methods may be easily employed to distinguish between them. Importantly, the rDNA maximum likelihood tree (Fig. 2) confirmed our notion that *S. frederici* does exist on the British Isles. First, worms recovered from British *A. sylvaticus* formed separate deeply divided clades with 100% bootstrap support, separated from the *S. obvelata* clade, confirming two separate species. Our isolates of putative *S. frederici*, whether from Nottingham, Berkshire or Wales, formed a tight clade with worms known to be *S. frederici* from Poland and Portugal (see Materials and Methods). The rDNA sequence of the worm from Portugal differed by just a single SNP. Hence, known samples of European *S. stroma* and *S. frederici* clustered within the same clade as the worms from British hosts that are suspected of belonging to the same species. In the case of worms, we had assigned to *S. stroma* based on location in their hosts and morphological criteria, there was little variation in amplified rDNA sequences, one SNP in each of the Portugal (13As16Ss) and Scotland worms (06As52Ss) in the ITS-2 region. Second, as hypothesized, worms recovered from the small intestine fell within the *S. stroma* clade, while worms confined to only the caecum formed a clade containing only *S. frederici*.

The CO1 primers amplified a 792 bp region across all species differing only by SNPs. In cases where the 'reference identification' is the same between trees, the gene was amplified from cDNA belonging to the same worm. Amplification of this region was not as reliable as the rDNA region resulting in a different set of isolates used for the phylogenetic analysis, although consistency was maintained where possible. The maximum likelihood phylogeny produced four distinctive clades (Fig. 3) containing *S. stroma*, *S. obvelata*, *S. frederici* from *A. sylvaticus*, with the fourth clade containing *S. frederici* from *Apodemus speciosus* (Okamoto *et al*. 2007). Importantly, this tree supports the rDNA phylogeny, although with a greater level of within

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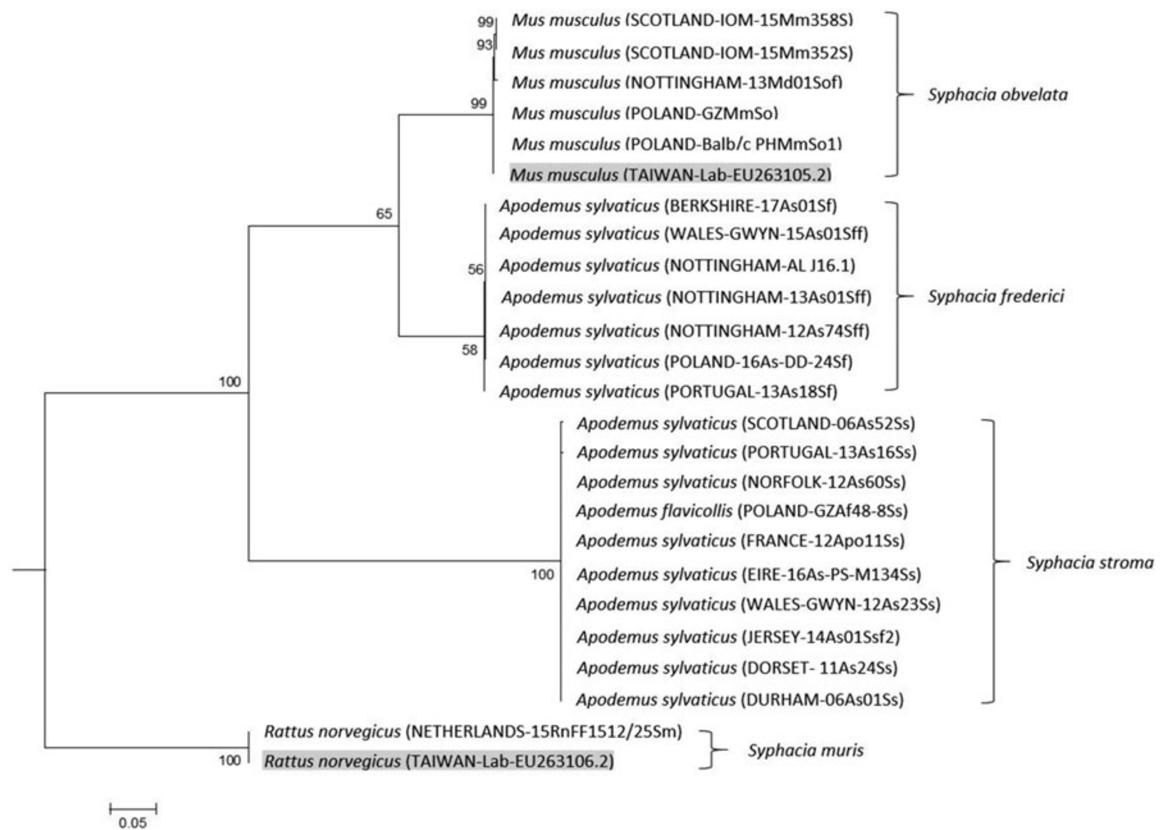

Fig. 2. Molecular phylogenetic tree of *Syphacia* from murid hosts (*Apodemus* spp. and *Mus* spp.) based on the ribosomal DNA following maximum-likelihood analysis with 100 bootstrap replicates implemented *via* the RAxML package. Scores at nodes represent bootstrap support for that node. Scale bar is proportional to the genetic distance in substitutions per site. Highlighted isolates are based on sequences from GenBank.

species variation, and demonstrates that *S. stroma* and suspected British *S. frederici* form their own deep individual clades, with *S. frederici* also grouping with a known European sample. Interestingly, the Japanese *S. frederici* forms its own distinctive clade from that of European *S. frederici* with 100% bootstrap support, the result of 45 unique SNPs between the two clades across the 792 bp region. GenBank sequences for three other species, *Syphacia emileromani*, *Syphacia ohtaorum* and *Syphacia agraria*, indicated that these species are unrelated to the *S. stroma*, *S. obvelata* and *S. frederici* clades.

Morphological comparison of worms

Key specimens were studied morphologically as highlighted in Table 1, with comparative measurements given in Table 3. A consistent difference in external morphology between *S. stroma* and *S. frederici* is in the shape of the tail of female worms. As illustrated in Figs 4 and 5, the end of the tail in female *S. stroma* is not as fine and attenuated as that of *S. frederici*, being rather broader and tapering to an end more sharply. In *S. frederici*, the tapering is more gradual, resulting in a longer, thinner and finer tip to the tail. Moreover, the tail in female *S. frederici* is often bent or twisted as shown in Figs 4B, 4C and 5. The morphology of the tail of female *S. stroma* worms was well illustrated by Morgan (1932), while the comparison of both species is illustrated in Tenora and Mészáros (1975), and the twisted fine tapering tail of *S. frederici* was emphasized by Ryzhikov *et al.* (1979) and illustrated also in Sharpilo (1973). Other differences that can be observed in female worms using light microscopy include the presence of cervical and lateral alae in *S. frederici* but only cervical alae in *S. stroma*, and the vulva nearer the anterior end in *S. frederici*. On the whole, *S. frederici* also has smaller eggs although there is some overlap between the species and authors differ about the degree of overlap. Based on our measurements, eggs <112 μm in length are likely to be *S. frederici*, and those >112 μm are likely to be *S. stroma*. This is in general agreement with egg measurements recorded by Morgan (1932), Roman (1945), Sharpilo (1973), Baruš *et al.* (1979), Sharpilo (1973) and Ryzhikov *et al.* (1979). Baruš *et al.* (1979) also detail some morphological differences between the eggs of these and other species of *Syphacia* that can be seen under scanning electron microscopy (SEM), but these are not suitable for quantitative studies. It is worth pointing out here

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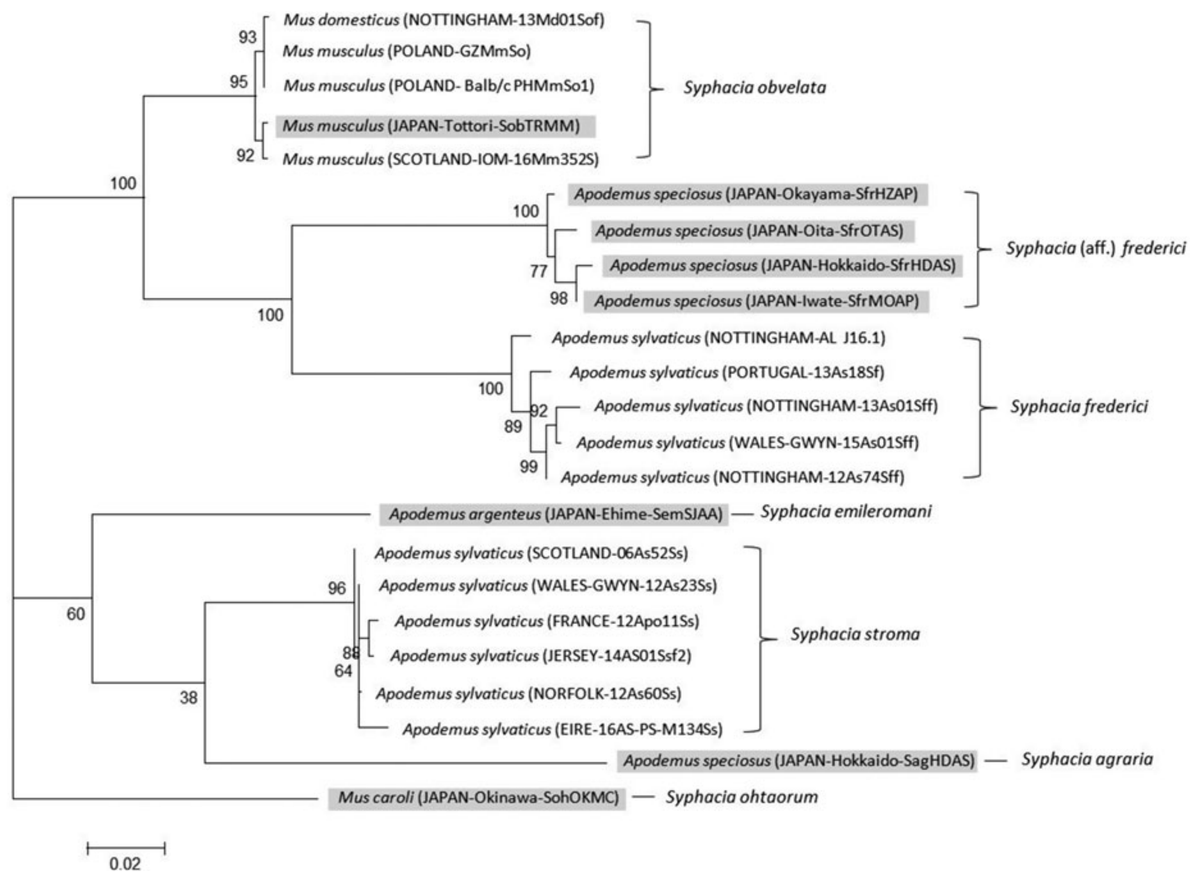

Fig. 3. Molecular phylogenetic tree of *Syphacia* from murid hosts (*Apodemus* spp. and *Mus* spp.) based on the mitochondrial cytochrome C oxidase 1 gene (CO1) following maximum-likelihood analysis with 100 bootstrap replicates implemented *via* the RAxML package. Scores at nodes represent bootstrap support for that node. Scale bar is proportional to the genetic distance in substitutions per site. Highlighted isolates are based on sequences from GenBank and all others are new sequences generated in the course of this study. *Syphacia ohtaorum* is described in Hasegawa (1991), *Syphacia emileromani* in Chabaud *et al*. (1963) and *Syphacia agraria* in Sharpilo (1973).

that our observation of the presence of cervical alae on *S. stroma* differs from Wiger *et al*. (1978) who concluded from a scanning electron microscopy (SEM) examination of *S. stroma* that this species has no cervical alae. However, Bernard (1966) described cervical alae for *S. stroma*, and was cited by Quentin (1971), and Morgan (1932) illustrated cervical alae on *S. stroma* although he did not refer to them as such.

There are also differences between the males with *S. federici* having shorter spicules (55–65 μm in *S. frederici* and 71–87 μm in *S. stroma* based on Ogden, 1971; Tenora and Meszaros, 1975; Ryzhikov *et al*. 1979). Unfortunately since males are not often found in infections with *Syphacia*, this may not be a useful discriminatory character. Quentin (1971), in his analysis of the species of *Syphacia* based on the morphology of the cephalic plate, placed *S. frederici* in Group VI (with a laterally thinner cephalic plate) and *S. stroma* in Group IX (with an oval cephalic plate), and these differences are easily recognizable in lateral view at higher magnifications. Wiger *et al*. (1978) using SEM were able to document another difference between the species. *Syphacia frederici* has a row of denticles on each of the three teeth, *S. stroma* does not. Similarly these characters are best viewed in *en face* preparations, at higher magnifications or by SEM and are not suited for quantitative studies. Wiger *et al*. (1978) also reported that *S. frederici* has longitudinal septa on the body surface, *S. stroma* does not and again these are best observed by SEM.

DISCUSSION

The primary objective of the study reported herein was to ascertain whether *S. frederici* exists in the wood mouse population of the British Isles, this species never having been recorded previously in wood mice from this region, and on the evidence we have presented we can confirm that this species does indeed parasitize wood mice in the British Isles. Over several decades of working on helminths in wild British rodents, we had occasionally observed wood mice with *Syphacia* sp. confined to the caecum, a characteristic of *S. frederici*, rather than the widely reported *S. stroma* which lives predominantly in the upper small intestine (Lewis,

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Table 3. Comparative measurements of females of *Syphacia frederici* and *Syphacia stroma* from *Apodemus sylvaticus* from localities in Poland, Portugal and the UK

	Range in mm (mean)	
	S. frederici	*S. stroma*
Length, mm	2·4–4·3 (3·05)	2·2–4·6 (3·4)
Width	153–221 (182·2)	144–429 (235)
Oesophagus length	268–368 (294·6)	288–489 (398·5)
Oesophagus bulb length	75·9–109 (89·6)	45–120·6 (86·75)
Oesophagus bulb width	69·3–95·7 (84·3)	48–115·5 (84·5)
Nerve ring	92·4–149 (110)	–
Excretory pore	205–590 (391·8)	502–603 (509)
Vagina to anterior	304–872 (581)	640–1360 (876)
Tail length	360–650 (506·4)	300–576 (427)
Egg length	102–132 (116·7)	112–160 (142·7)
Egg width	26·4–39·6 (32·3)	28·8–48 (40·1)

These measurements are based on all the worms that were measurable from among the isolates that were examined morphologically (for *S. frederici*: five worms from NOTTINGHAM-12As74Sff, 10 worms from WALES-GWYN-15As01Sff, five worms from PORTUGAL-13As21Sf, three worms from PORTUGAL-13As18Sf and five from POLAND-16As-DD-24Sf and for *S. stroma* eight worms PORTUGAL-12Apo3Ss, and worms at the BMNH registration numbers 1934·7·19·10–15; 1983. 3928–3929; 1956·8·16. 3–6; 1970. 63–68; 1970. 55–62;1979 175–194).

1968). Our molecular data show clearly that the *Syphacia* we included in the study fall into very distinct genetic clades, whether assessed by rDNA (ITS-1, 5·8S, ITS-2) or by the mitochondrial gene CO1 and further demonstrate that both our British suspected *S. frederici* isolates and worms known to be *S. frederici* from the European mainland constitute a single clade.

It is also of some interest that the CO1 gene, as sequenced from our specimens of *S. frederici* from the British Isles and Europe clearly differed from sequences published for *S. frederici* from *A. speciosus* in Japan (Okamoto *et al.* 2007 and GenBank, see Table 2), and these two formed two distinct clades with a similar evolutionary distance between them as, for example, *S. stroma*, *S. agraria* and *Syphacia emilromani* (Fig. 3). However, Hasegawa (1981) initially concluded that the female worms he recovered from *A. speciosus* in Japan were morphologically identical to the descriptions given by Roman (1945, 1951) and Quentin (1971) for worms from *A. sylvaticus* in Europe. Subsequently, Hasegawa *et al.* (1994) concluded that the morphology of Japanese '*S.* aff. *frederici*' does not correspond precisely with that of the European type material; indeed Hasegawa *et al.* (1994) suggested that the Japanese material should belong in a different subgenus to that conventionally assigned to European

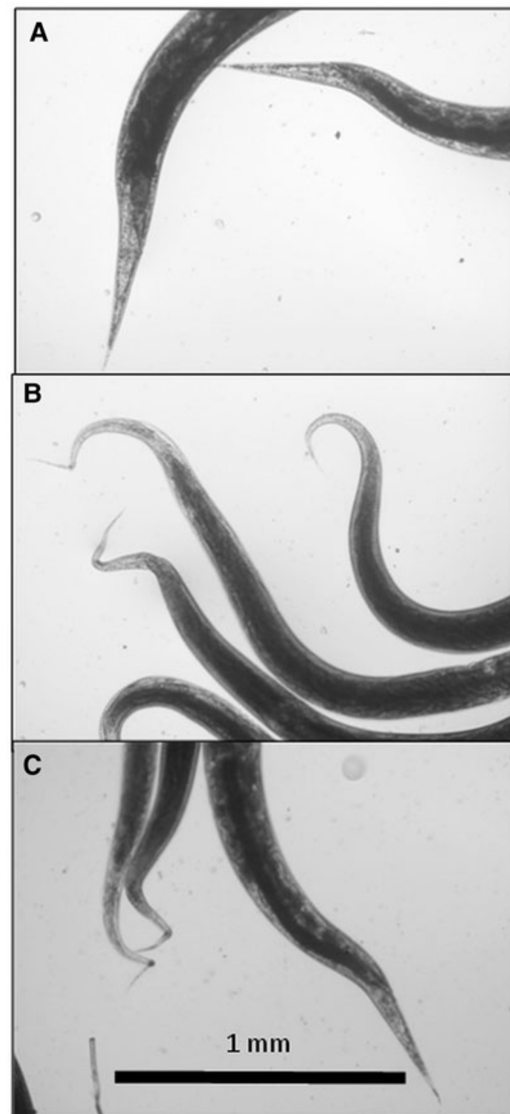

Fig. 4. The distal ends of female worms showing the difference in tails. (A) *Syphacia stroma*; (B) *Syphacia frederici*; (C) two *S. frederici* on the left and *S. stroma* on the right. The scale bar is 1 mm.

specimens. The eastern Asian nematode fauna differs in many respects to that found in western Europe (e.g. the genus *Aspiculuris*, see Behnke *et al.* 2015, or *Heligmosomoides*, see Zaleśny *et al.* 2014), and there is no *a priori* reason to suspect that *Syphacia* from Japan and western Europe should be conspecific. Moreover, *A. speciosus*, the host of *S.* aff. *frederici* in Japan, is a member of the subgenus *Apodemus*, whereas the type host of *S. frederici*, *A. sylvaticus*, is a member of the subgenus *Sylvaemus*; these subgenera diverged c. 8 million years ago (Michaux *et al.* 2001), giving ample time for speciation of their parasites to have occurred. A thorough morphological comparison of worms from Europe and Japan, especially at the SEM level, with molecular analysis of nuclear loci, is therefore necessary to resolve the specific identity of the Japanese worms.

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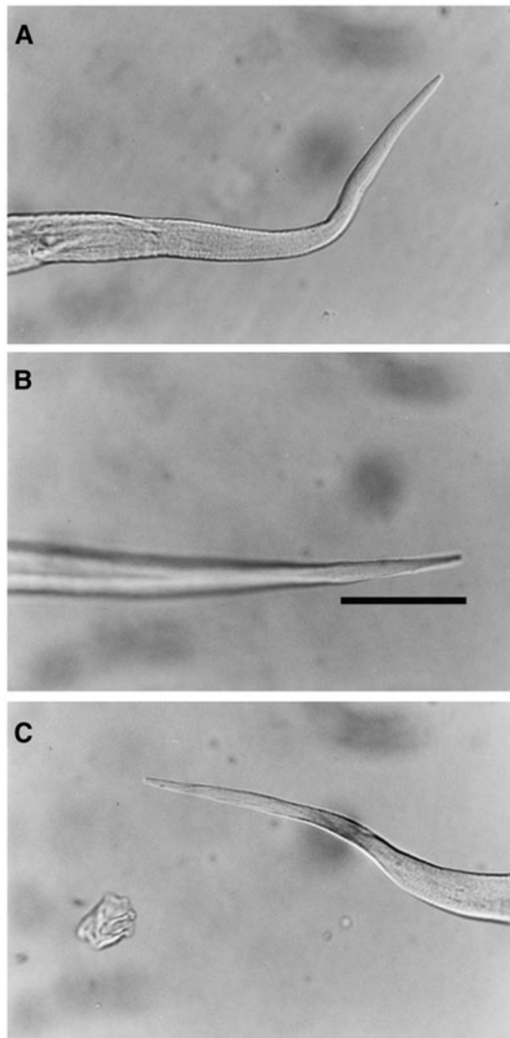

Fig. 5. The distal ends of three female *Syphacia frederici* from isolate WALES-GWYN-15As01Sff, to show the location of the point at which the tail bends. The bent tail in A and C are clearly apparent, and in B while the tail is straighter, most likely a fixation artefact, the point of flexion is clearly visible and suggests that an investigation of the ultrastructure of this feature may be revealing. All images were taken at the same magnification with ×20 objective. Scale bar in B is 10 μm.

Taken together, our genetic data are consistent with the idea that *S. frederici*, which was originally described from French wood mice (Roman, 1945), exists in the UK population of *A. sylvaticus*. Based on both CO1 and rDNA, the gene sequences were either identical or very similar to those of worms from continental mice, from a region where *S. stroma* does not exist (Urwitałt in the Mazury region of Poland – JMB & AB pers. obs.) and from other locations where *S. frederici* is known to exist (Portugal; see Eira *et al.* 2006). All the *S. frederici* isolates used for genetic analysis, whether from British wood mice or from abroad, were female worms recovered from the caeca of their respective hosts.

Morphological inspection confirmed that the *S. frederici* identified in this study conformed to the morphological descriptions of the species provided by authors in the past. Individuals of *S. frederici* can be readily distinguished from *S. stroma* as described above. Where specimens are in good condition, cervical and lateral alae can be detected easily. Moreover, the differences in the cephalic end of each species can also be seen easily. The morphology of the female tail is particularly useful in this regard allowing the screening of large numbers of individual worms, at relatively low microscopical magnification, for identification, even in circumstances where mixed infections are present.

Thus, on three lines of evidence (location in the caecum and not small intestine, phylogenetics, and morphology), we can confirm that *S. frederici* does indeed parasitize wood mice in the British Isles, as it does on the European mainland. The interesting question that arises now is why *S. frederici* has never previously been reported from wood mice in the British Isles, despite the many thorough studies of helminth communities in the region?

One obvious explanation is that morphologically at low magnification *S. frederici* and *S. stroma* are actually superficially quite similar to one another in appearance, and to distinguish between them based simply on morphology takes some experience and is time consuming. Moreover, much of the taxonomy of *Syphacia* spp. is based on male worms, which dominate in available keys to the genus (see, e.g. Tiner's, 1948 and Khera's, 1956 keys to the genus), primarily because females, which together with juveniles usually form the vast majority of the worm burden in infected animals, are relatively poor in characters suitable for species discrimination, difficult to distinguish between and in some species even indistinguishable morphologically. Hence understandably, female worms have been given far less attention in keys. However, male worms which are believed to die soon after mating are very small, very rare and in some species unknown (Morgan, 1932; Lewis, 1968; Abdel-Gaber, 2016).

Syphacia stroma was the first described of the two species. In the early literature of the genus, the specific status of *S. stroma* was not accepted (e.g. in Seurat, 1916, the first paper using the name *Syphacia*, *S. stroma* is clearly considered a junior synonym of *S. obvelata*). This led to confusion that persisted throughout the earlier reports on wood mouse helminths in which wood mice were reported to be infected with *S. obvelata*, the typical *Syphacia* sp. of house mice (Seurat, 1915; Elton *et al.* 1931; see Ogden, 1971). Infections with *S. stroma* can be very intense, running to many hundreds and even thousands of worms, and often swamp numerically other concurrently infecting helminths (Lewis, 1968; Behnke *et al.* 1999; Abu-Madi *et al.* 2000). To check each worm microscopically is a hugely onerous task, although several authors working on the European continent have successfully

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differentiated these two species quantitatively in *Apodemus* spp. populations that were exposed to both (Eira *et al*. 2006; Milazzo *et al*. 2010; Čabrilo *et al*. 2016). In very heavy infections, *S. stroma* can also spill into the caecum of its host, and even in low-intensity infections, female worms migrate through the caecum to lay their eggs on the perianal surface of their host (Kerboeuf and Lewis, 1987).

As stated earlier, *S. frederici* was described more recently, and may therefore be less well known. Published during wartime, the original description is brief (eight lines) and deals only with the mature female and meristic characters of the eggs, as the male at that time was unknown. This original description is very poor providing the reader with little to go on in terms of distinction from other species. It was not until the reviews by Quentin (1971), and Tenora and Meszaros (1975), and chapters in books by Sharpilo (1973) and Ryzhikov *et al*. (1979) that some of the key differences between *S. frederici* and *S. stroma* were more clearly reported, but even from the information provided in these it is not easy to extract the essential, workable differences between the species, and translations of the latter two Russian texts (both are in Cyrillic) are lacking. *Syphacia* are in any case character-poor, and many morphological differences identified between *S. stroma* and *S. frederici* are too difficult to implement in quantitative studies (e.g. those based on SEM) in which hundreds/thousands of worms may be recovered from individual hosts and need to be identified to species level. Two differences, however, are easier to implement in quantitative studies; the location of the majority of the worms in the caecum in the case of *S. frederici* and in the small intestine in the case of *S. stroma* and, as we have shown here and with some experience, the fine morphology of the tails in female worms can be distinguished even under low magnification microscopy. Further, if conducting phylogenetic analysis of this genus for species determination, we recommend the rDNA region, which is easier to amplify in a standard PCR.

To date, no *S. frederici* have been detected in Ireland, based on the molecular analysis of *Syphacia* in this study and lack of observations of *Syphacia* infections solely occurring in the caecum in previously reported helminth surveys and current ongoing studies (personal Communication; I. Montgomery, P. Stuart and K. Loxton). However, despite no earlier reports of its presence in Britain, it is possible that *S. frederici* has parasitized British wood mice for as long as these rodents have existed on the British mainland, but has simply not been recognized as a different species to *S. stroma*. Alternatively, *S. frederici* may have been introduced to the British Isles more recently, although given that some ecological studies of wood mouse helminths in Britain were conducted as recently as the 1990s without reporting *S. frederici* (Abu-Madi *et al*. 2000; Behnke *et al*. 1999), it seems unlikely that this species could have spread as widely in the last 3–4 decades as between the locations referred to in this paper (i.e. Nottingham in the centre and Berkshire in the central south of the British Isles, and Anglesey in the north-west tip of Wales). It may of course be more widespread, or indeed show a patchy distribution across the British Isles since *Apodemus* populations in the British Isles and Ireland originated in a variety of ways, including from potential southern refugia (Montgomery *et al*. 2014) and from synanthropic transfer with colonizing or invading humans (Berry, 1973; Yalden, 1999), and their parasites originated with them; *Heligmosomoides polygyrus* of two quite distinct mitochondrial haplotypes occur within the British Isles, for example, one widespread in western Europe, while the other has been found only in Britain, Ireland and Denmark (Nieberding *et al*. 2005; Cable *et al*. 2006; Zaleśny *et al*. 2014). A heterogeneous distribution of *Syphacia* species might therefore be expected, but further relevant fieldwork must be conducted to elucidate these patterns. In this context, the distribution of small indels in the sequence of ITS-2 of *Syphacia* species may prove invaluable in tracking the fine-grained phylogeography of their hosts.

Finally, this study highlights further the potential cryptic or 'near-cryptic' diversity of parasitic nematodes, even within such well-studied host groups as European rodents. By highlighting the two easily examined key differences between *S. frederici* and *S. stroma*, it is hoped that this study will make it much easier for future studies in the British Isles to confirm with confidence the presence/absence of S. *frederici* in wood mice from England and Wales, and to ascertain whether wood mice in Ireland and Scotland also carry this species.

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