Bank voles (*Myodes glareolus*) and house mice (*Mus musculus musculus; M. m. domesticus*) in Europe are each parasitized by their own distinct species of *Aspiculuris* (Nematoda, Oxyurida)

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(*Received 13 April 2015; revised 22 June 2015; accepted 22 June 2015*)

SUMMARY

The molecular phylogeny and morphology of the oxyuroid nematode genus *Aspiculuris* from voles and house mice has been examined. Worms collected from *Myodes glareolus* in Poland, Eire and the UK are identified as *Aspiculuris tianjinensis*, previously known only from China, while worms from *Mus musculus* from a range of locations in Europe and from laboratory mice, all conformed to the description of *Aspiculuris tetraptera*. Worms from voles and house mice are not closely related and are not derived from each other, with *A. tianjinensis* being most closely related to *Aspiculuris dinniki* from snow voles and to an isolate from *Microtus longicaudus* in the Nearctic. Both *A. tianjinensis* and *A. tetraptera* appear to represent recent radiations within their host groups; in voles, this radiation cannot be more than 2 million years old, while in commensal house mice it is likely to be less than 10 000 years old. The potential of *Aspiculuris* spp. as markers of host evolution is highlighted.

Key words: *Myodes glareolus*, *Clethrionomys glareolus*, *Chionomys nivalis*, *Aspiculuris*, *Mus musculus musculus*, *Mus musculus domesticus*, bank voles, house mice.

INTRODUCTION

The oxyuroid nematode *Aspiculuris tetraptera* (Nitzsch, 1821) is a well-known contaminant of laboratory mice, a pest that is easily transmitted in conventional animal houses and a major headache for animal house staff breeding specific pathogen-free mice for research projects (Flynn, 1973; Taffs, 1976). It is also well known as a parasite of wild house mice *Mus musculus* Linnaeus, 1758 (both *Mus musculus musculus* and *M. m. domesticus* Rutty, 1772; see Suzuki *et al.* 2013), occurring widely in Europe (e.g. UK, Denmark, Germany, Austria, Czech Republic, Serbia; see Behnke, 1975; Sage *et al.* 1986; Moulia *et al.* 1991; Kataranovski *et al.* 2008; Baird *et al.* 2012) and throughout the world (Hugot, 1980; Tattersall *et al.* 1994; de Bellocq *et al.* 2012).

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Aspiculuris tetraptera has also been recorded from a range of other rodent species, including wood mice (*Apodemus sylvaticus* Linnaeus, 1758), but infrequently in this host and usually only as a few worms, suggesting incidental infections (e.g. Bernard, 1987; Ryan and Holland, 1996; Behnke *et al.* 2001; de Bellocq *et al.* 2003). However, several authors, including ourselves, have reported the worm from European bank voles [*Myodes* (= *Clethrionomys*) *glareolus* (Schrieber, 1780)] (Sharpe, 1964; Lewis, 1987; Behnke *et al.* 2008; Bjelic-Cabrilo *et al.* 2011), not just as incidental infections, but showing high prevalences and with heavy worm burdens, indicating frequent transmission among individuals of this host species and a capacity to mature and survive to, and beyond, patency (Thomas, 1953; Sharpe, 1964; Lewis, 1987). For some time we have suspected that the worms from bank voles and house mice may represent different species, despite their superficial morphological similarity. The ecology of their host species is quite different, with little overlap of frequented territories.

Parasitology (2015), **142**, 1493–1505.
doi:10.1017/S0031182015000864

In most parts of the world, including Europe, house mice are mainly anthropophilic, living in close proximity to villages, farms and other human habitation, although in Australia they have adapted to colonize agricultural land on which cereals are grown and eruptions of plague proportion occur at regular intervals (Singleton *et al*. 2005). On the other hand, red-backed voles of the genus *Myodes* Pallas, 1811 are predominantly woodland animals and while they may occasionally be trapped in cultivated fields and may even venture into buildings, these species do not overlap extensively in their territories with house mice, their diets differ and they are rarely likely to encounter one another in the wild (Flowerdew *et al*., 1985; Bujalska and Hansson, 2000). Although some degree of gene flow cannot be ruled out, nevertheless, it is not easy to see how a species such as *A. tetraptera* could remain panmictic as a major parasite of rodents in both *Mus* and *Myodes*, a key requirement if these taxa are not to diverge and eventually speciate. Evolutionary theory predicts that ecological separation should eventually lead to genetic separation and separate species status (Coyne and Orr, 2004).

It may also be pertinent that nematodes of the order Oxyurida Weinland, 1858, are renowned for their host specificity and are well recognized as having undergone co-evolution with their hosts. Some of the best evidence for co-evolution of parasites with their hosts is derived from the tightly congruent host–parasite phylogenetic trees that have been generated using both morphological and genetic criteria (Enterobiinae; Hugot, 1999). Based on data such as these, it would be remarkable therefore if the worms from *M. glareolus* and *M. musculus* were conspecific. Evidence that the species of *Aspiculuris* Schultz, 1927 from bank voles may be different from that infecting house/laboratory mice is also provided by an attempt to infect wild-caught bank voles with fully embryonated and infective eggs isolated from worms recovered from laboratory mice: the eggs given to bank voles failed to survive to maturity with larvae persisting for fewer than 10 days, while those given to laboratory mice developed normally (Behnke, 1974).

While records of species of *Aspiculuris* from *Apodemus* appear incidental, two other species of the genus have been described from voles; *Aspiculuris dinniki* Schultz, 1927, from the snow vole *Chionomys nivalis* (Martins, 1842) (see Schulz, 1927) from the northern Caucasus in Russia, and *Aspiculuris tianjinensis* Liu *et al*. 2012, recently reported as parasitizing the grey-sided vole *Clethrionomys rufocanus*, now known as the grey red-backed vole *Myodes rufocanus* (Sundevall, 1846) in China (Liu *et al*. 2012). Neither of these two *Aspiculuris* spp. is well known, and neither has been subjected to molecular genetic analysis. In this paper, we tested the null hypothesis that *A. tetraptera* from bank voles and from house/laboratory mice are the same species. First, we compared specimens of *Aspiculuris* recovered from bank voles (*M. glareolus*) and from house and laboratory mice (*M. m. musculus* and *M. m. domesticus*), using conventional light and scanning electron microscopy, with a focus on the key morphological features that are known to vary between species within the genus. We also compared our material to descriptions of *A. tetraptera* and *A. tianjinensis* in the literature. We then amplified and sequenced a fragment of the nuclear ribosomal RNA gene spanning the 5·8S rDNA molecule, the Internal Transcribed Spacer 2 region (ITS2) and the mitochondrial Cytochrome Oxidase 1 (CO1) gene, generating a phylogeny of *Aspiculuris* for the first time to demonstrate the relationship between isolates from house and laboratory mice, bank voles and other rodent hosts that were available to us (Table 1).

MATERIALS AND METHODS

Nomenclature and terminology

In this paper, nomenclature for bank voles (the genus *Myodes*) follows Carleton *et al*. (2003, 2014) and not Tesakov *et al*. (2010) and for all other rodents Musser and Carleton (2005). Terminology used to describe the cephalic end and cervical alae of species of *Aspiculuris* follows Inglis *et al*. (1990) and Hugot (1980).

Sources of Aspiculuris

Specimens of *Aspiculuris* were collected from *M. musculus* (both laboratory cultures and from wild mice) and from the arvicolids *Microtus duodecimcostatus* (de Selys-Longchamp, 1839), *C. nivalis* and *M. glareolus* from various locations in Europe (Tables 1 and 2). As outgroup material for the molecular analysis we included *Aspiculuris africana* Quentin, 1966 from the spiny mouse *Acomys dimidiatus* (Cretzschmar, 1826) from the Sinai in Egypt (Behnke *et al*. 2004) and *Aspiculuris americana* Erickson, 1938 from *Peromyscus leucopus* (Rafinesque, 1818) (Erickson 1938) from Pennsylvania, USA (Table 2). We also included worms collected from *Microtus longicaudus* (Merriam, 1888) from Montana, USA. All pertinent details of hosts, parasites and collection localities are given in Tables 1 and 2, which include also the specific reference codes employed by ourselves and our collaborators for worms in their collections. Rodents were collected according to the legal and ethical guidelines current in the countries where they were sampled.

Methods utilized for morphological comparison of species

Individuals of *Aspiculuris* were recovered from the large intestines of voles and mice, the intestines

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Table 1. Details of the worms, *A. tetraptera*, *A. tianjinensis* and *A. dinnicki* used in the current study for morphometrics

Species and Reference of isolate	Number and sex	Host	Source
Aspiculuris tetraptera			
SCOTLAND 10MmMGA3Atm	10 males	Laboratory mice PARTON strain	Edinburgh, Scotland
NOTTINGHAM 12Md11-12Atm1 and 13Md 01-Atf2	10 males + 10 females	*M. m. domesticus*	Tollerton, Nottingham (pig farm)
Aspiculuris dinniki			
SWITZERLAND 14MnAd2	4 males	*C. nivalis*	Lausanne, Switzerland
Aspiculuris tianjinensis			
POLAND 10Mg275Atfa	10 females	*M. glareolus*	Mazury, Poland
POLAND 10Mg275Atma	10 males	*M. glareolus*	Mazury, Poland
SCOTLAND 0906Atf	1 female	*M. glareolus*	Edinburgh, Scotland
SCOTLAND 0908Atf	2 females	*M. glareolus*	Edinburgh, Scotland
EIRE-GCa26-11	3 females	*M. glareolus*	Coole Park, Galway, Eire
EIRE-GCa26-11	2 males	*M. glareolus*	Coole Park, Galway, Eire
EIRE- GM 29-11	3 females	*M. glareolus*	Merlin Park, Galway, Eire
EIRE- GM 29-11	3 males	*M. glareolus*	Merlin Park, Galway, Eire
WALES-1107Atm	6 males	*M. glareolus*	Anglesey, Wales
WALES-12Mg25At	1 male	*M. glareolus*	Anglesey, Wales
WALES-12Mg25At	8 females	*M. glareolus*	Anglesey, Wales
NOTTINGHAM 1142Atm	10 males	*M. glareolus*	University Park, Nottingham, England
NOTTINGHAM 1141AtfR	10 females	*M. glareolus*	University Park, Nottingham, England

having been preserved in 70% ethanol prior to dissection. The recovered worms were then frozen at −80 °C in 80% ethanol. Prior to microscopic examination, specimens were cleared in lactophenol for study as wet mounts. *En face* and transverse sections were prepared by hand cutting with a cataract scalpel and mounted in polyvinyl lactophenol. Figures were prepared with the aid of a drawing tube and measurements in micrometres, unless otherwise stated, were taken using an eyepiece micrometer and light micrographs were taken using an Olympus photomicrographic system. Representative specimens were fixed in glutaraldehyde and dehydrated in ethanol, followed by critical point drying and gold coating (Sputter Coater SCD-030, Balzers Union, FL9496), for viewing under scanning electron microscopy (Jeol JSH-840 Scanning Electron Microscope).

All specimens examined morphologically for this study, except those used for scanning electron microscopy, were deposited in the South Australian Museum, Adelaide with registration numbers SAM AHC47104–47108 and 47543–47546.

Molecular genetic comparison of species

DNA was isolated from individual worms using DirectPCR Lysis Reagent (Viagen Biotech), according to the manufacturer's instructions. DNA primers (Table 3) were used in 25 μL PCR reactions containing 12·5 μL of BioMix Red (Bioline), 0·5 μM forward primer, 0·5 μM reverse primer, <250 ng template DNA and nuclease-free water to a final volume of 25 μL. Thermal cycler conditions for the 18S ribosomal locus amplification were as described by Fontanilla and Wade (2008; 94 °C for 2 min, then 38 cycles of 94 °C for 30 s, 45 °C for 30 s and 65 °C for 1 min). For ITS2 primer reactions were: 98 °C for 2 min, 35 cycles of (98 °C for 15 s, 61 °C for 15 s, 72 °C for 15 s) and 72 °C for 5 min. Thermal cycle and times for CO1 primer reactions were: 98 °C for 2 min, 35 cycles of (98 °C for 15 s, 60 °C for 20 s, 72 °C for 20 s) and 72 °C for 5 min. All PCR reactions were conducted in a Biorad PTC-200 DNA Engine Cycler. PCR products were visualized on 1·5% agarose gels, incorporating ethidium bromide to a final concentration of 0·5 μg mL^{-1}, then purified (ExoSAP, Affymetrix), and DNA concentration estimated (Nanodrop), before dilution with nuclease-free distilled water. Sequencing primers (identical to amplification primers) were added to the appropriate concentration prior to Sanger sequencing (Source Bioscience) and chromatograms inspected visually to resolve ambiguities.

Alignments were produced using ClustalX within the Mega 6.0 package (Tamura *et al*. 2011) followed by visual inspection. Phylogenetic analysis was conducted using a maximum-likelihood algorithm implemented in RaxML version 8.0 (Stamatakis, 2014) via the CIPRES Science Gateway portal (Miller *et al*. 2010). Analysis of the ribosomal sequences was undertaken using the full sequences (including indels) with *A. africana* and *A. americana*

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Table 2. Details of the worms used in the current study for genetic analysis

Reference	Host[a]	Source	Putative parasite
SCOTLAND-0903At	*M. glareolus*	Edinburgh, Scotland	*A. tianjinensis*
SCOTLAND-0907At	*M. glareolus*	Edinburgh, Scotland	*A. tianjinensis*
NOTTINGHAM-1142Atf	*M. glareolus*	Nottingham, England	*A. tianjinensis*
NOTTINGHAM-1141Atf	*M. glareolus*	Nottingham, England	*A. tianjinensis*
WALES-1102At	*M. glareolus*	Anglesey, Wales	*A. tianjinensis*
WALES-1111Atm	*M. glareolus*	Anglesey, Wales	*A. tianjinensis*
WALES-0902At	*M. glareolus*	Anglesey, Wales	*A. tianjinensis*
WALES-1111Atf	*M. glareolus*	Anglesey, Wales	*A. tianjinensis*
NORFOLK-12Mg63At	*M. glareolus*	Norfolk, England	*A. tianjinensis*
POLAND-14Mg21Atf	*M. glareolus*	Mazury, NE Poland	*A. tianjinensis*
POLAND-08-680Atfm	*M. glareolus*	Mazury, NE Poland	*A. tianjinensis*
POLAND-14Mg174At	*M. glareolus*	Mazury, NE Poland	*A. tianjinensis*
EIRE-12Mg28-12	*M. glareolus*	Galway, Eire	*A. tianjinensis*
EIRE-10SPMgAt	*M. glareolus*	Galway, Eire	*A. tianjinensis*
USA-Mkin-2014-43Aaf	*M. longicaudus*	Montana, USA	*A. dinniki*
SWITZERLAND-14Mnad3	*C. nivalis*	Valais mountains, Switzerland	*A. dinniki*
SWITZERLAND-14Mnad4	*C. nivalis*	Valais mountains, Switzerland	*A. dinniki*
PORTUGAL -12microAt	*M. duodecimcostatus*	Pancas, Portugal	*A. dinniki*
FRANCE-14-060603 ms	*A. sylvaticus*	French Pyrenees	*A. tetraptera*
SPAIN-13RIBASAt	*M. m. domesticus*	Les Franqueses del Valles, Catalonia, Spain	*A. tetraptera*
POLAND-14Mm02At	*M. m. musculus* (CBA)	Lublin, Poland	*A. tetraptera*
GERMANY-SK2365	*M. m. domesticus*	Bavaria, Germany	*A. tetraptera*
GERMANY-SK2324	*M. m. domesticus*	Bavaria, Germany	*A. tetraptera*
POLAND-BALB/c-10MmWroUniatf	*M .m. musculus* (BALB/c)	Wrocław, Poland	*A. tetraptera*
GERMANY-14-AS2333	*M. m. domesticus*	Schrotzhofen, Germany	*A. tetraptera*
GERMANY-14-AS2353	*M. m. domesticus*	Eichlberg, Germany	*A. tetraptera*
GERMANY-14-AS2306	*M. m. domesticus*	Furth, Germany	*A. tetraptera*
BRAZIL-13MmAt	*M. m. musculus* (LAB)	Fortaleza, Ceará State, Brazil	*A. tetraptera*
SCOTLAND-PARTON-10MmAt	*M. m. musculus* (Parton)	Edinburgh, Scotland	*A. tetraptera*
NOTTINGHAM-13Md01Atm	*M. m. domesticus*	Nottingham, England	*A. tetraptera*
EGYPT-12Ad268Aa	*A. dimidiatus*	Sinai, Egypt	*A. africana*
USA-11PlAam	*P. leucopus*	Pennsylvania, USA	*A. americana*

[a] For *Mus* hosts, the strain of mouse is given in parenthesis if the parasites were derived from laboratory mice or (LAB) is given if uncertain. All other records from *Mus* hosts are from wild-caught animals

Table 3. Primers for the amplification of specific products as used in the phylogenetic analysis

Primer name	Primer sequence
18S Forward	5′ AAAGATTAAGCCATGCATG 3′
18S reverse	5′AGCTGGAATTACCGCGGCTG 3′
ITS2F	5′ CTCTTAGCGGTGGATCACTCGGC 3′
ITS2R	5′ CAAAGTTCTTTGCAACTTTCCC 3′
CO1F	5′ GGTTTTTTGGTCATCCTGAGG 3′
CO1R	5′ CAAAACCAACAGTAAACATATG 3′

as outgroups; using an alignment omitting the outgroups (minimizing indels); and excluding all indels and ambiguous regions. None of these changes made any difference to the final tree, which is presented based on the full sequences plus outgroups. Reference sequences for *A. tetraptera* from GenBank were included in the alignment, as were sequences from *A. tetraptera* collected from Chinese mice (details below in Results section). The CO1 sequences were checked as inferred amino acid sequences to ensure that there were no frame shifts or unlikely amino acid substitutions in the fragment. Voucher sequences have been deposited in GenBank (CO1 – KT175702 to KT175724; ITS2 – KT175725 to KT175737).

RESULTS

Morphological examination of Aspiculuris

The worms from laboratory mice (Parton strain) from Edinburgh, Scotland (Reference = SCOTLAND 10MmMGA3Atm, 10 males and 10 females) and those from wild mice in Nottinghamshire, England

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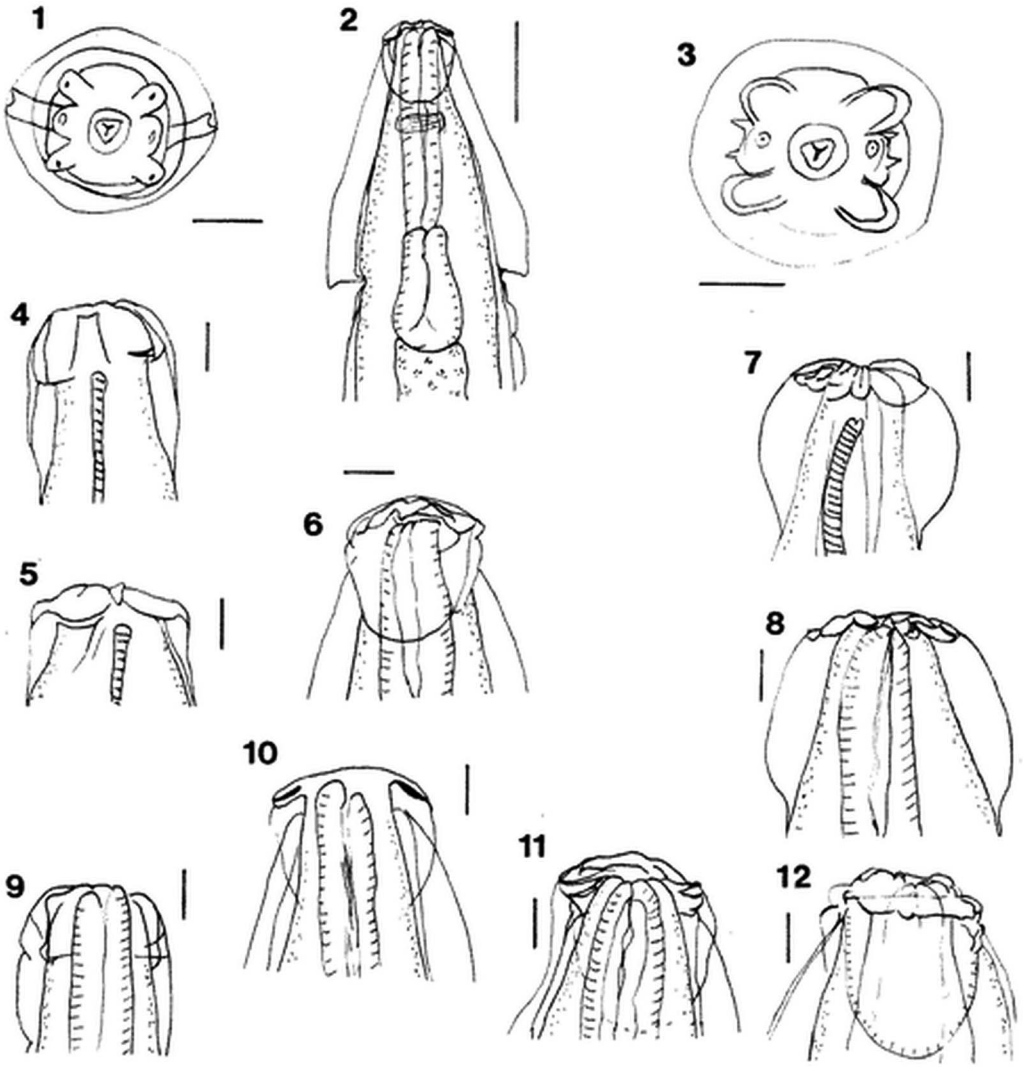

Fig. 1. *Aspiculuris tetraptera* from *Mus musculus* and *Aspiculuris tianjinensis* from *Myodes glareolus*. 1.1 – *A. tetraptera* female *en face* view; 1.2 – *A. tetraptera* male ventral view anterior end; 1.3 – *A. tianjinensis* male *en face* view; 1.4 – *A. tetraptera* male lateral view surface aspect; 1.5 – *A. tetraptera* female lateral view surface aspect; 1.6 – *A. tetraptera* male dorso-ventral view surface aspect; 1.7 – *A. tianjinensis* female lateral view surface aspect; 1.8 – *A. tianjinensis* female lateral aspect optical section; 1.9 – *A. tianjinensis* female lateral view optical section; 1.10 – *A. tetraptera* male dorso-ventral view optical section; 1.11 – *A. tianjinensis* male lateral view optical section; 1.12 – *A. tianjinensis* male lateral view surface aspect. Scale bars: 1, 4–12, 25 μm; 2, 100 μm; 3, 20 μm.

(Reference = NOTTINGHAM 12Md11-12Atm1, 10 males and 10 females), all conformed to the accepted descriptions of *A. tetraptera* (see Hugot, 1980).

Examination of four males of *Aspiculuris* from snow voles from Switzerland showed that morphologically they conformed to the description of *A. dinniki* (see Schulz, 1927), for which the type host is *C. nivalis*. They could be easily distinguished from *A. tetraptera* in having 10 and not 12 caudal papillae and a single pair of caudal alae that do not reach the tail tip, rather than 3 pairs of alae, the third pair reaching the tip of the tail, as is typical of *A. tetraptera*.

Examination of 32 male and 37 female *Aspiculuris* from bank voles from England, Poland, Scotland, Ireland and Wales (Table 1) showed that morphologically they conformed to the description of *A. tianjinensis* as reported by Liu *et al.* (2012).

Morphological comparison of Aspiculuris *spp. from bank voles and house/laboratory mice*

Aspiculuris tetraptera and *A. tianjinensis* differ morphologically in the details of the distinct, elaborate inflated region formed by an anterior cephalic cap bearing lateral amphids, dorso-ventral cephalic papillae and lobed posterior extensions of the dorsal and ventral surfaces of the body, that together make up the cephalic vesicle (Inglis *et al.* 1990). This complex of structures is difficult to interpret and describe but can be more clearly illustrated (Figs 1–3). Both species have the typical small

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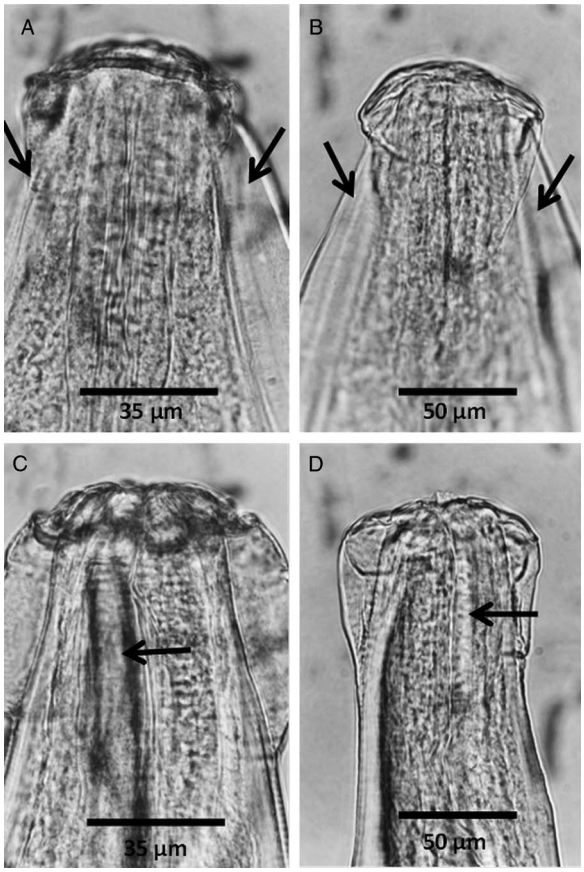

Fig. 2. Light microscope images of the cephalic ends of *Aspiculuris tianjinensis* from *Myodes glareolus* from Wales (A, C) and *Aspiculuris tetraptera* from *Mus musculus* from Scotland (B, D), showing dorso-ventral views (A, B) and lateral views (C, D). Arrows indicate cephalic alae. Scale bars: A, C – 35 µm; B, D – 50 µm.

hexagonal mouth opening without lips (Figs 1.1, 1.3 and 3) surrounded by 6 small sessile labial papillae. The differences are seen in the proportions of the four dorso-ventral cephalic papillae and the 2 lateral epaulettes with amphids on the anteriormost level (Figs 1–3). These are larger and more robust in *A. tianjinensis* than *A. tetraptera*. Moreover, the proportions of the lateral cap are relatively smaller in *A. tianjinensis*, such that the lobes of the cephalic papillae extend over the edges of the cap, whereas in *A. tetraptera* the lobes of the cephalic papillae do not extend over the edges of the cephalic cap (Figs 1 and 3). The cervical alae of *A. tianjinensis* appear to begin more posteriorly to the cephalic cap than those of *A. tetraptera* although this small difference may be due to the specimens examined having not been processed using identical protocols. The males of both species have 12 caudal papillae, 5 pairs and 2 median; however, the median papillae are double in *A. tetraptera* and single in *A. tianjinensis*, the post-cloacal papilla being relatively larger in *A. tetraptera* (see Hugot, 1980; Liu *et al.* 2012). The cervical alae terminate more abruptly in *A. tetraptera* (ending about the mid-oesophageal bulb) than *A. tianjinensis* (ending nearer to the oesophageal junction) although the actual lengths of the alae overlap. Accurate determination of the relative positions of alae and mid-oesophageal bulb is likely to be influenced by fixation of the worms and whilst efforts were made to keep this consistent between the two species, some variation was inevitable when processing material collected and fixed in the field, and in examining material supplied by collaborators for this project (see Acknowledgements for list).

Based on Liu *et al.* (2012) the Chinese specimens of *A. tianjinensis* are larger than the English, Irish, Scottish, Welsh or Polish specimens of *A. tianjinensis* collected from bank voles for this study and they are also larger than the English specimens of *A. tetraptera* studied. There are no clear differences in morphometrics between the two species (see Table 4 for comparative measurements).

Molecular genetic comparison of Aspiculuris *spp.*

Molecular analyses of *Aspiculuris* from house mice were entirely consistent with previous analyses of *A. tetraptera*. The ribosomal primers amplified a fragment of 1024 bp from all isolates (laboratory or wild) extending through 5·8S rDNA and ITS-2 into 28S rDNA, which was identical to the reference sequences (NCBI accession EU263107 and EF464551) from laboratory mouse colonies deposited by Parel *et al.* (2008) and Feldman (unpublished). Across a common stretch of 416 bp the consensus sequence differed by single nucleotide polymorphisms (SNPs) from four Chinese sequences in GenBank (NCBI accession KJ143618 A27G; KJ143617 T207C and G417A; KJ143616 T377C; KJ143615 G417A; Lou *et al.* 2015). The fragment amplified from *Aspiculuris* from voles varied in length between 1022 bp (*M. longicaudus*) and 1028 bp (*M. glareolus*), a difference primarily due to a TG microsatellite at the beginning of the 28S rDNA gene. The sequences from worms from *P. leucopus* (*A. americana*) and *A. dimidiatus* (*A. africana*) were highly divergent; the former was 1050 bp in length, the latter 1005 bp. A maximum-likelihood phylogeny showed a fundamental split between *A. americana* from *P. leucopus* and *A. africana* from *A. dimidiatus*, and the *A. tetraptera*-like forms from voles and mice (Fig. 4). The latter also showed a deep split between the forms from house mice (referable to *A. tetraptera*, with 100% bootstrap support) and the forms from voles (referable to *A. tianjinenensis* and *A. dinniki* based on morphology, which grouped with 87% bootstrap support). Maximum-likelihood analysis of aligned sequences from which indels and ambiguous regions had been deleted gave an identical phylogeny, with slightly improved bootstrap support for the major clades. The worms from *M. duodecimcostatus*, *M. longicaudus* and *C. nivalis* grouped together with

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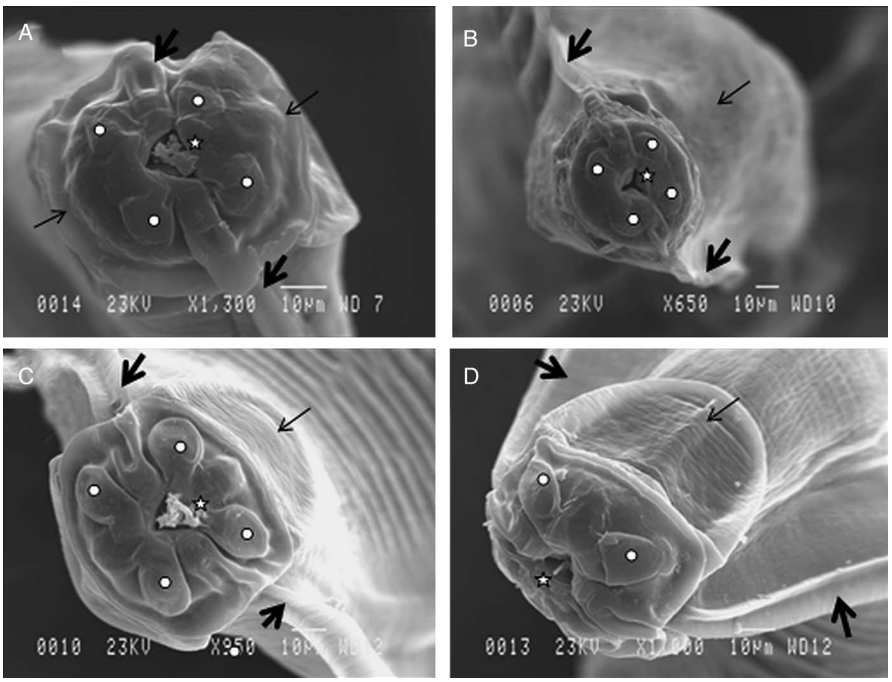

Fig. 3. Scanning electron microscope images of the anterior ends of representative samples from bank voles and from house/laboratory mice. (A) *En face* view *Aspiculuris tetraptera* from wild house mouse from England. (B) *En face* view *A. tetraptera* from laboratory mouse from Poland. (C) *En face* view *Aspiculuris tianjinensis* from bank vole England. (D) Dorso-ventral view *A. tianjinensis* from bank vole England. The large lobes of the cephalic vesicle can be clearly seen. Bold black arrows point to cervical alae and their epaulettes as they connect with the cephalic vesicle; thin black arrows indicate the inflated regions on the dorsal and ventral surfaces of the worms as relevant; the white circles indicate each of the 4 cephalic papillae; white star indicates the dorsal surface in each image.

strong bootstrap support (79%); forms from *C. nivalis* were morphologically identical to *A. dinniki*, and were collected from the type host. Tentatively we refer worms from *Microtus* Schrank, 1798 to *A.* cf *dinniki*, pending additional research on the variability of *Aspiculuris* from this vole genus. A single worm was available from *A. sylvaticus* collected from Spain, together with material from *M. musculus* in the same locality. Interestingly, at this ribosomal locus this worm represented a sister group to the *A. tetraptera* cluster infecting *Mus*: the ribosomal fragment was 1022 bp in length and differed at 3 SNPs, and by a total of 3 GT repeats at microsatellites. While this is within the range of variation for this locus observed in isolates from voles, it does exceed that seen within worms from *Mus*.

Amplification of the Small Subunit ribosomal RNA locus generated a fragment 515 bp long from worms from both *Mus* and *Myodes*. These fragments differed by a single SNP at position 198 in the alignment, which was a C in worms from *Mus* and a T in worms from *Myodes*. Lacking other SNPs, and lacking sequences from worms from *C. nivalis*, *Microtus* spp. or *A. sylvaticus*, this locus was not included in the alignment used for phylogenetic analysis.

Amplification at the CO1 locus was not as reliable as ITS-2, and a different set of isolates were used for this phylogeny. The 189 bp fragment contained 18 SNPS; three resulted in changes to the inferred amino acid sequence, and further variation was confined to synonymous substitutions. All amplicons from worms from *Myodes* had identical sequences, as did all PCR products from worms collected from *Mus*. The broad trends in the two phylogenies were similar with a clear cut difference between the sequences from voles and mice (Fig. 5). However, while the *A. africana* CO1 sequence differed by 3·5–5% from all other isolates, the *A. americana* sequence was only 1% different from the *Aspiculuris* sequences from voles. While the two principal groupings, worms from *Mus* (*A. tetraptera*) and worms from *Myodes* (*A. tianjinensis*) were both recreated in the CO1 phylogeny, only the clade (*A. dinniki sensu stricto*) containing worms from *Chionomys* (Satunin, 1909) was also supported (bootstrap support 77%), and at all higher levels bootstrap support was insufficient to imply relationship. The worms from *Microtus* spp. (*A.* cf *dinniki*) did not group with each other or with *A. dinniki*, but generally bootstrap support was so low that the significance of their failure to form a single clade cannot be assessed.

DISCUSSION

The molecular phylogenies based on available *Aspiculuris* material make it clear that the worms

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Table 4. Comparative morphometrics of *A. tetraptera* and *A. tianjinensis*, data from our study, Hugot (1980) and Liu *et al.* (2012).

	A. tianjinensis Current study Males Poland $n = 8$	*A. tianjinensis* Current study Females Poland $n = 10$	*A. tianjinensis* Liu *et al.* (2012) Males China $n = 7$	*A. tianjinensis* Liu *et al.* (2012) Females China $n = 12$	*A. tetraptera* Hugot (1980) Males Venezuela $n > 100$	*A. tetraptera* Hugot (1980) Females Venezuela $n > 100$	*A. tetraptera* Current study Males Nottingham $n = 10$	*A. tetraptera* Current study Females Nottingham $n = 9$
Length (mm)	2–3	4–4·5	3·69–5·12	5·38–7·0	2·7	3·8	1·6–3·7	3·5–4·5
Width	153–170	204–289	204–388	282–447	110	200	136–238	167·5–272
Cephalic vesicle length	39·4–62·7	66–89·1	69–78	88–93	80		67–99	92·4–113·9
Cephalic vesicle width	52·8–79·2	99–115·5	88–122	113–162	90		68–102·3	93·6–113·9
Oesophagus length	264–316·8	330–412·5	343–379	417–480	380	430	301–408·7	395·3–502·5
Oesophageal bulb length	99–122	115·5–138·6	74–142	123–167	140	160	105·5–148·5	132–184·4
Oesophageal bulb width	66–89·1	92·4–105·6	59–123	88–137	90	130	66–89·1	82·5–115·5
cervical alae length	264–323·4	330–402	284–338	382–441	260	350	244–368·5	321·6–402
Nerve ring	99–125·4	108·9–148·5	181–245	121–157	100	130	132–148·5	99–145·2
Excretory pore to anterior	422·2–603	742·5–1122	930–1030	1090–1230	700	900	742·5–891·8	770–1005
Tail	148·5–204·6	809–958	181–245	920–1140	170	550	167·5–207·7	402–643·2
Vulva to anterior		1407–3655						1273–1700
Eggs		79·2–89·1 × 42·9–49·5						82·5–92·4 × 36·3–49·5
Ovejector		198–295						165–297

All measurements are in micrometres, unless otherwise stated.

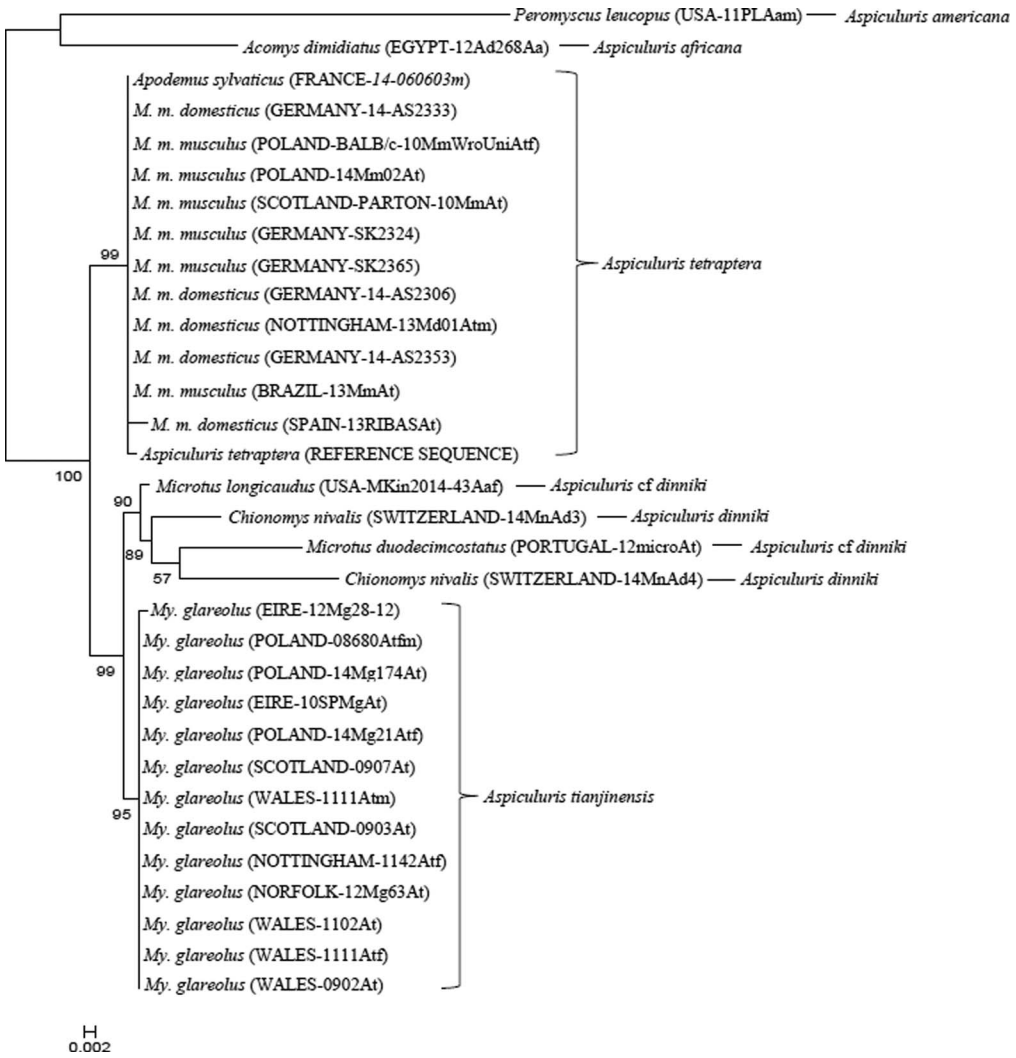

Fig. 4. Molecular phylogenetic tree of *Aspiculuris* from mice and voles based on the nuclear 5·8S and ITS-2 following maximum-likelihood analysis with 100 bootstrap replicates implemented via the RaXML package. Scores at nodes represent bootstrap support for that node. Outgroups are *Aspiculuris americana* from *Peromyscus leucopus* and *Aspiculuris africana* from *Acomys dimidiatus*. Scale bar is proportional to the genetic distance in substitutions per site.

from the bank vole, *M. glareolus*, are distinct from both *A. dinniki* from the snow vole, *C. nivalis*, and from *A. tetraptera* from the house mouse. The molecular distance between the worms from voles and *A. tetraptera* is such that there can be no suspicion that these are recently diverged forms, due to isolation caused by the ecological differentiation of their hosts. Rather it is clear that *Aspiculuris* has radiated recently within Holarctic voles of the genera *Microtus* and *Myodes*, and that the greater diversity of the genus as currently understood, occurs within these hosts. We identify the material from *M. glareolus* with *A. tianjinensis*, based on morphological evidence as provided in the original description (Liu *et al*. 2012), because material of the latter taxon from eastern China was not available for either morphological or molecular study.

The genus *Aspiculuris* was created by Schulz (1924) to accommodate *A. tetraptera*, originally described as *Ascaris tetraptera* by Nitzsch (1821) and transferred into Rudolphi's genus *Oxyura* by Diesing (1861). Descriptions and drawings from the 1830s (e.g. Schmalz, 1831) make it absolutely clear that this taxon described in 1821 as *Asc. tetraptera* was identical to that currently recognised as *A. tetraptera*. By the mid-1970s, when the genus had grown to include a number of heterogeneous species, Quentin (1975) recognized the importance of the shape of the anterior cervical alae, either arrowhead-shaped or spear-shaped in species discrimination (Quentin, 1975; Inglis *et al*. 1990). Four of the species in the present study *A. dinniki, A. tianjinensis, A. tetraptera* and *A. americana* all have arrowhead-shaped alae, while the fifth, *A. africana* has spear-shaped alae. Hugot (1980) used developmental evidence to support the argument that species with spear-shaped outlines were more primitive. Our molecular ITS-2 phylogeny makes it clear that *A. americana*, from *P. leucopus* and *A. africana* from *A. dimidiatus* are the most divergent species included in our analysis, and the arrowhead alae do not group with a monophyletic clade in the genus.

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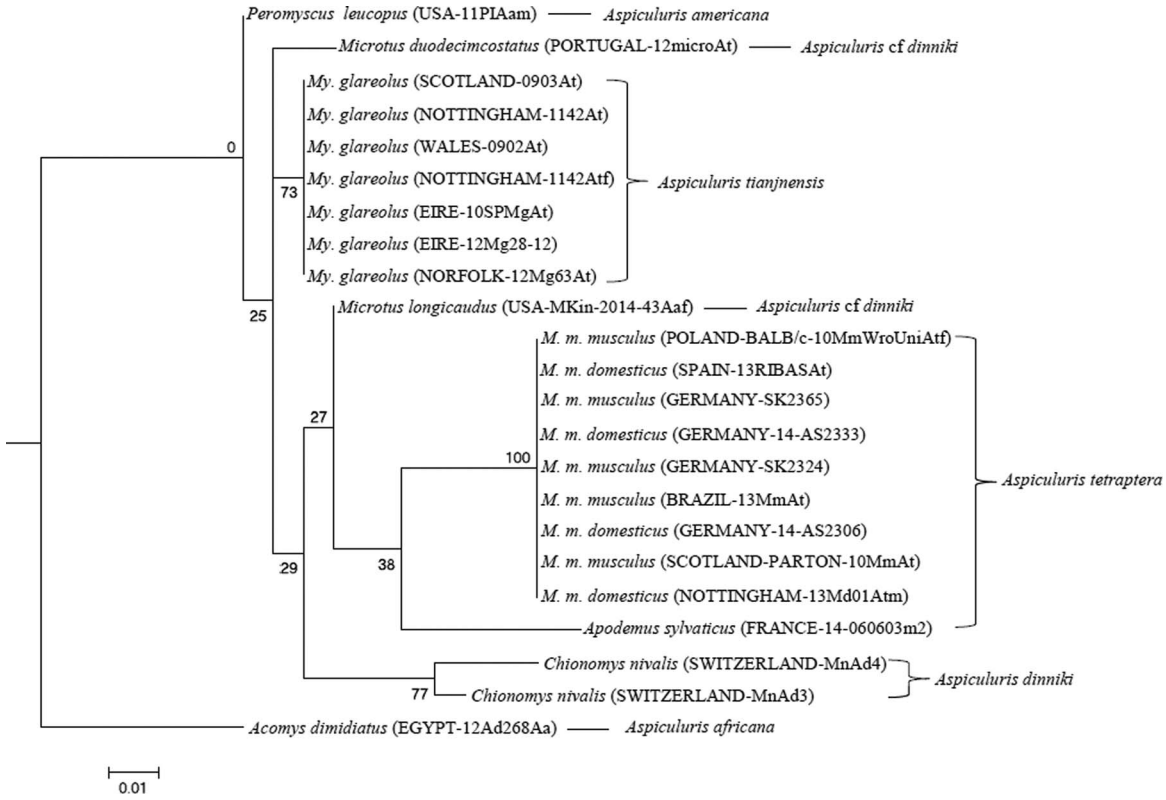

Fig. 5. Molecular phylogenetic tree of *Aspiculuris* from mice and voles based on the mitochondrial Cytochrome Oxidase 1 gene (CO1) following maximum-likelihood analysis with 100 bootstrap replicates implemented via the RaXML package. Scores at nodes represent bootstrap support for that node. Scale bar is proportional to the genetic distance in substitutions per site.

Therefore, the morphology of the cervical alae cannot be considered at this time as reliable evidence of relationship and consequently alternative evidence is required to determine whether Quentin's (1975) division of the genus into four primitive species with continuous cervical and lateral alae (a spear-shaped anterior outline) and a larger group of species with discontinuous alae (an arrowhead-shaped anterior outline) is valid. Additional molecular analysis of a greater range of species, particularly those with a spear-shaped anterior outline, is required to fully resolve the relationship between these morphological features and evolutionary relationships between species.

In the past, the morphological identification of species of *Aspiculuris* has been based primarily on the relative length and shape of the cervical alae as for example in the key of Akhtar (1955) and the number and configuration of the caudal papillae of male worms. However, this has raised confusion because both of these character sets can be misinterpreted if the effects of fixation are not taken into account. In particular, not all caudal papillae are readily detectable when the ventral caudal area has become curled or creased during fixation. Furthermore, it may be difficult to see the median papillae and to decide whether these are single or double as described by Hugot (1980). Consequently we believe that some authors have miscounted the number of caudal papillae for these species. As redescribed by Hugot (1980), *A. tetraptera* has 5 pairs of single papillae and 2 double papillae and we were able to confirm this with our specimens. These can be counted either as 12 (5 × 2 + 1 + 1) or as 14 (5 × 2 + 2 + 2) papillae. *A. tianjinensis* has 12 papillae, made up of 5 pairs and 2 single median papillae which do not appear to be double (Liu *et al.* 2012). Our specimens from bank voles also had 12 papillae, with the two median papillae not doubled, matching exactly the description given by Liu *et al.* (2012). Comparisons of the *en face* and lateral aspects of the cephalic ends of specimens have proved to be more reliable characters for separating the species than either morphology of the alae or numbers of caudal papillae for these two species. Both Hugot (1980) and Inglis *et al.* (1990) have provided useful descriptions of the complex of cephalic structures that are found within the genus, allowing species discrimination even where morphometrics are similar.

It is clear from the current work that *A. tetraptera* and *A. tianjinensis* are not sister taxa undergoing incipient speciation, but are only distantly related despite their superficial morphological resemblance. However, it does appear that *A. tianjinensis* and *A. dinniki* are more closely related, and that these are part of a wider radiation of *Aspiculuris* within the recently evolved Arvicolinae. While records of *Aspiculuris* from *Apodemus* appear incidental, several

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Aspiculuris spp. have been described from voles; two, *A. dinniki*, from the snow vole *C. nivalis* (see Schulz, 1927), and *A. tianjinensis*, recently reported as parasitizing *M. rufocanus* in China (Liu *et al.* 2012), and recorded here as widespread in *M. glareolus* in Central Europe, are fairly well documented. *A. kazakstanica* Nasarova and Sweschnikowa, 1930, a parasite of voles from central Asia, is known only from the secondary Soviet Russian literature, and is a species that requires re-assessment and detailed confirmation of the original description. It is especially interesting to note that whereas these species, or *A. tetraptera* s.l., are widely recorded from voles in the Soviet Russian host-parasite literature (e.g. Rishikov, 1979), the only records of the genus from voles in North America are those of an '*A. tetraptera*'-like form from *M. longicaudus and M. gapperi* recorded by Kinsella (1967; and see also Doran, 1955), although *A. americana* occurs in the neotomine rodent *Peromyscus* Gloger, 1841 in the Nearctic. We have been able to include worms from *M. longicaudus* in our molecular analysis, and can confirm these as related to *A. dinniki,* from *Chionomys,* forming a single clade at the ribosomal locus with *A. dinniki sensu stricto* and with worms from *M. duodecimcostatus* (Fig. 4). We identify these worms as *A.* cf *dinniki*, but a reappraisal of the taxonomy of *Aspiculuris* from *Microtus* and its allies is clearly necessary. It is likely that worms in this clade are *Microtus* and *Chionomys* specialists, these host genera being closely related. The colonization of Northern America by both *Microtus* and *Myodes* is very recent, and for example the North American *M. gapperi* (Vigors, 1830) has been suggested to be paraphyletic with respect to both the Eurasian *M. glareolus* and the holarctic *M. rutilus* Pallas, 1779 (see Cook *et al.* 2004), taxa which have probably diverged since the early Pleistocene, circa 2 million years ago (MYA). While multilocus sequencing with a larger dataset throws doubt on this paraphyly (Kohli *et al.* 2014), there is no doubt that the origin of the American *Myodes* is recent; Kohli *et al.* (2014) estimate the date for the divergence of *M. glareolus* and *M. gapperi* (presumably a Eurasian vole) as 1·25 MYA ± 0·75 million years. The origin of the North American *Microtus* species, including *M. longicaudus*, is obscure, but is also unlikely to be as much as 2 MYA (Chaline *et al.* 1999; Jaarola *et al.* 2004). It appears then that the *Aspiculuris* fauna of North America contains representatives of at least two radiations, the older in neotomine rodents while the second in voles is more recent.

The only murid which is regularly infected with *Aspiculuris* in Eurasia is *M. musculus* and although several species are known from *M. musculus* from other geographical regions (e.g. *A. huascensis* Falcon-Ordaz *et al.* 2010 and *A. lahorica* Akhtar, 1955), these appear closely related to *A. tetraptera*, if in fact they do indeed represent distinct species (Akhtar, 1955; Falcon-Ordaz *et al.* 2010). The commensal rat, *Rattus rattus* Linneaus, 1758, is also infected by *A. pakistanica* (Akhtar, 1955), but this species is very similar to *A. tetraptera* in morphology (Akhtar, 1955). Reports of *A. tetraptera* from rats usually indicate only low prevalence and intensity, and mostly from study sites frequented also by sympatric populations of house mice (Milazzo *et al.* 2010). *A. tetraptera* has been reported from *Mus spretus* Lataste, 1883 in Spain and Portugal, the latter in an environment where house mice also abounded, so these were probably also incidental infections from house mice (Behnke *et al.* 1993; Fuentes *et al.* 2000; Sainz-Elipe *et al.* 2007). *A. shikouleta*, a species with alae continuous along the sides of the body, the spear-shaped profile, has been recorded from the distantly related African murid *Micaelamys namaquensis* (Smith, 1943) reported as *Aethomys namaquensis* by Inglis *et al.* (1990). However, the only other records of *A. tetraptera*-like worms from murids are the sporadic reports of this parasite from *Apodemus*. Our data show clearly on molecular genetic evidence that these worms from *Apodemus* are similar to *A. tetraptera* but not identical to it, being distinctly different from worms from *Mus* at both ITS-2 and CO1 loci. There are no records of *A. tetraptera*-like worms from any other murid, despite the popularity of these rodents for parasite taxonomic and faunistic surveys. There is certainly no evidence of close co-evolution between *Aspiculuris* and murid rodents in the way that has been described for species of *Syphacia* Seurat, 1916 (see Hugot, 1988; but see Okamoto *et al.* 2007). The status of *Aspiculuris* from *Apodemus* should be reviewed further, with material collected from throughout the geographical and taxonomic range of wood mice in case these should prove to be distinct from *A. tetraptera*. The most likely explanation for the occurrence of *A. tetraptera* in *Mus* is a host switch into house mice at the time when the latter first became abundant commensals with the rise in agriculture some 10 000 years ago, an explanation similarly advanced to account for the infection of mice by *Heligmosomoides bakeri* (Durette-Desset *et al.* 1972; Nieberding *et al.* 2006) (see Behnke and Harris, 2010). The origin of the worms involved in this host switch is unknown, but several *Aspiculuris* species with arrowhead cervical alae are known from other cricetid rodents in similar habitats to those in which the host switch probably took place (Rishikov, 1979).

The current work lends support to the experimental observations that *A. tetraptera*, from laboratory mice cannot mature in bank voles in experimental cross-infections (Behnke, 1974). We also predict that *A. tianjinensis* and *A. tetraptera* cannot interbreed, and they cannot be regarded as sibling or sister species. At first sight, the speciation of *Aspiculuris* in commensal murids is spectacular, if the timings suggested in this paper are correct;

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indeed, the divergence of a taxon such as *A. huascensis* must be very rapid since house mice did not reach Mexico until the relatively recent historical past, and there are no described examples of *Aspiculuris* with arrowhead alae from native Central or South American rodents. Indications of such a divergence may be simply the result of lack of sampling effort in the region. Alternatively, if genuine, divergence on the time scale proposed here may owe much to the unusual breeding biology of oxyuroid nematodes. Oxyuroids have a haplodiploid reproductive mechanism (Adamson, 1989, 1994), with haploid males developing from unfertilised eggs. This, coupled with auto-reinfection as a result of grooming could lead to a high frequency of back-crossing and fixation of minor morphological variants such as appear to be represented by *A. huascensis* and *A. lahoricus*. At the same time, this breeding biology makes *Aspiculuris* and *Syphacia* highly resistant to environmental change, and these two parasites are the most likely to be translocated successfully with their host mice under the most rigorous conditions (for example, in Iceland or on some sub-Antarctic islands; Skirnisson *et al.* 1993; Pisanu *et al.* 2003). Clearly, aspicularid nematodes could prove highly useful markers for the rapid evolution of both arvicoline and murine rodent evolution, when molecular markers from a sufficiently wide range of populations have been taken into consideration.

ACKNOWLEDGEMENTS

We thank Dr Mike Kinsella for his guidance and inspiration, and for provision of specimens from the USA, and Prof. Peter Vogel (who sadly died in January 2015 before the completion of our ms) and Charlotte Vogel, Switzerland for samples from *C. nivalis* from which we recovered *A. dinniki*. We are also grateful for the efforts of Dr. Keisuke Nakata, Japan for samples from *M. rufocanus* which unfortunately did not yield *Aspiculuris* and to Russian ecologists and parasitologists who also endeavoured to find for us *A. tianjinensis* from *M. rufocanus*. We thank the following colleagues for specimens sent to us at various times during our project: Sarah Perkins, Celia Holland and Karen Loxton for specimens from Eire, Claudia Bevilaqua for specimens from laboratory mice from Brazil, Grzegorz Zaleśny for specimens from Wrocław and Joelle Gouy de Bellocq for help in collecting samples from Germany. We also thank John Lewis and Celia Holland for their comments on earlier drafts of this paper. Technical assistance and expertise with scanning electron microscopy was provided by Denise McLean, in the Advanced Microscopy Unit (School of Life Sciences, University of Nottingham).

FINANCIAL SUPPORT

We are grateful to the Universities of Nottingham and Warsaw for financial support for travel and consumables. Our work was supported additionally by travel grants from the Royal Society (JMB), the British Ecological Society (JMB), the Grabowski Fund (JMB), the Polish State Committee for Scientific Research (KBN, AB), and by the KBN and British Council's Young Scientist Programme (AB). VKJ was supported in part by NIH (grant number R21-A11076631).

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