Molecular and morphological circumscription of *Mesocestoides* tapeworms from red foxes (*Vulpes vulpes*) in central Europe

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SUMMARY

Here we examine 3157 foxes from 6 districts of the Slovak Republic in order to determine for the first time the distribution, prevalence and identity of *Mesocestoides* spp. endemic to this part of central Europe. During the period 2001–2006, an average of 41·9% of foxes were found to harbour *Mesocestoides* infections. Among the samples we confirmed the widespread and common occurrence of *M. litteratus* (Batsch, 1786), and report the presence, for the first time, of *M. lineatus* (Goeze, 1782) in the Slovak Republic, where it has a more restricted geographical range and low prevalence (7%). Using a combination of 12S rDNA, CO1 and ND1 mitochondrial gene sequences together with analysis of 13 morphometric characters, we show that the two species are genetically distinct and can be differentiated by discrete breaks in the ranges of the male and female reproductive characters, but not by the more commonly examined characters of the scolex and strobila. Estimates of interspecific divergence within *Mesocestoides* ranged from 9 to 18%, whereas intraspecific variation was less than 2%, and phylogenetic analyses of the data showed that despite overlapping geographical ranges, the two commonly reported European species are not closely related, with *M. litteratus* more closely allied to North American isolates of *Mesocestoides* than to *M. lineatus*. We confirm that morphological analysis of reproductive organs can be used to reliably discriminate between these often sympatric species obtained from red foxes.

Key words: *Mesocestoides*, *Vulpes vulpes*, 12S rDNA, CO1, ND1, Slovakia.

INTRODUCTION

Over the last 2 decades red fox (*Vulpes vulpes*) populations in Europe have increased substantially as a result of successful rabies vaccination campaigns and possibly better food resources. Expanding populations and their encroachment upon urban areas presents an increasing public health hazard for the human population (Švrček *et al*. 2000). Among the helminths hosted by European foxes, the occurrence of the tapeworm *Echinococcus multilocularis* has been monitored most intensively over the last decade (e.g. Pétavy *et al*. 1990; Duscher *et al*. 2006; Hegglin *et al*. 2008; Kinčeková *et al*. 2008; Miterpáková *et al*. 2003, 2006, 2009). Another common, but less-studied tapeworm group transmitted by these hosts is represented by the genus *Mesocestoides* Vailant, 1863 (Cestoda, Cyclophyllidea, Mesocestoididae), which are also parasites of dogs and other carnivores (Rausch, 1994) and have been reported in at least 27 cases of human infection (Fuentes *et al*. 2003).

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Mesocestoides spp. are unique among tapeworms in several aspects of their biology, including their life cycles, that are thought to involve 3 hosts (Etges, 1991; Rausch, 1994) and include a larval stage called a tetrathyridium. These presumed second-stage larvae show little host specificity and have been reported from the peritoneal cavity and parenchymal organs of a large diversity of mammals, birds and reptiles (Specht and Voge, 1965; Loos-Frank, 1980*a*; McAllister *et al*. 1991; Millán *et al*. 2003; Literák *et al*. 2004). Unlike foxes and humans, dogs, and to a lesser extent cats, are known to serve as both definitive and intermediate hosts (Crosbie *et al*. 1998, 2000; Thiess *et al*. 2001; Caruso *et al*. 2003; Toplu *et al*. 2004; Foronda *et al*. 2007; Wirtherle *et al*. 2007; Eleni *et al*. 2007), and as the definitive hosts they could be involved in the transmission cycle.

Morphologically, the group is unique in exhibiting a median ventral position of the genital atrium and a bipartite vitelline gland and, unusually, possessing a paruterine organ, rarely found in other cyclophyllidean groups (see Georgiev and Kornyushin, 1994). On this basis they have been traditionally classified in their own family, Mesocestoididae Fuhrmann, 1907, within the order Cyclophyllidea (Khalil *et al*. 1994). However, molecular analyses have demonstrated that *Mesocestoides* spp. represent 1 of 4

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primary lineages that together with the orders Cyclophyllidea, Nippotaeniidea and Tetrabothriidea, comprise the most derived clade of cestodes (termed 'higher acetabulates' *sensu lato* Olson *et al*. 2001, 2008) (Olson *et al*. 2001, 2008; Olson and Tkach, 2005; Waeschenbach *et al*. 2007). The interrelationships of these groups are poorly resolved (see Olson and Tkach, 2005) and it is thus unclear how close *Mesocestoides* is to the clade that represents the Cyclophyllidea. Nevertheless, independence of the *Mesocestoides* lineage clearly reflects the unique biology of the group and suggests that it should be recognized at an ordinal rank (i.e. Mesocestoidea) alongside other higher acetabulate groups.

Although ubiquitous and widespread across the Northern Hemisphere, *Mesocestoides* species are known to display an unusually high degree of phenotypic plasticity that has made routine species identification difficult without the aid of molecular tools, contributing to confusion regarding their species boundaries, geographical ranges and host associations (Voge, 1955; Loos-Frank, 1987; Padgett *et al*. 2005), and according to Rausch (1994), few species can be indentified on the basis of morphology alone. In Europe, 2 species of *Mesocestoides*, *M. litteratus* (Batsch, 1786) and *M. lineatus* (Goeze, 1782), have been reported commonly (Pétavy *et al*. 1990; Kapel and Nansen, 1996; Willingham *et al*. 1996; Okulewicz *et al*. 2005; Dalimi *et al*. 2006; Literák *et al*. 2006). According to Skrjabin (1978) and Jancev (1986), the two species may be differentiated morphologically by subtle differences in the cirrus sac, testes number and position of ovaries and vitellaria. However, such differences require careful microscopical examination of stained and mounted specimens and thus the species cannot be reliably differentiated in the field. Moreover, as in the present paper, most reports on European *Mesocestoides* are based on examination of previously frozen worms, which contributes to morphological variability and poor preservation of parasite specimens in general. However, this is a typically unavoidable consequence of the manner in which foxes are routinely surveyed and examined. We therefore believe that many previous reports of European *Mesocestoides* spp. are likely to have confounded the identities of these species.

Few molecular studies of the group have been conducted. Among European isolates, Nickisch-Rosenegk *et al*. (1999) reported a slight divergence in 12S rDNA among *Mesocestoides* spp. from red foxes in Southern Germany, and Literák *et al*. (2006) reported no divergence in 18S rDNA among isolates of *M. litteratus* from red foxes in Spain and the Czech and Slovak Republics. In North America, molecular-based studies of *Mesocestoides* spp. in dogs, coyotes, foxes and other animals (Crosbie *et al*. 1998, 2000; Padgett and Boyce, 2004; Padgett *et al*. 2005) revealed the existence of 3 highly distinct lineages that were neither host specific nor could they be distinguished on the basis of features of their scolex and strobila (see Padgett *et al*. 2005). Moreover, only 1 of the lineages could be assigned to a known species (i.e. *M. vogae* Voge, 1955 = *M. corti* Etges, 1991). Padgett *et al*.'s (2005) findings, together with morphological assessments of the group (Voge, 1955; Rausch, 1994), underscore the need to employ molecular data in the circumscription of these highly variable species (Olson and Tkach, 2005).

A broader sampling of European isolates, including molecular confirmation of *M. litteratus* and *M. lineatus*, has not been conducted previously. The Slovak Republic is a heavily mountainous and forested country in central Europe that is home to a large red fox population, and monitoring of these populations provided the opportunity to examine more than 3000 individuals collected over a 6-year period. Here we report on infections of *Mesocestoides* spp. in these populations and use a combination of 3 mitochondrial genes (12S rDNA, CO1 and ND1) and 13 morphometric characters to assess the validity of the commonly reported European species *M. litteratus* and *M. lineatus*.

MATERIALS AND METHODS

Necroscopy and examination of hosts

During the years 2001–2006, a total of 3157 red foxes (*Vulpes vulpes*) were examined from 6 administrative districts of the Slovak Republic: Bratislava (BA), Dolný Kubín (DK), Košice (KE), Nitra (NT), Prešov (PO) and Zvolen (ZV). All foxes were collected in Spring and Autumn and were shot by hunters according to government initiatives to manage population growth resulting from anti-rabies vaccination campaigns. Animals were transported to the State Veterinary and Food Institutes for rabies examination. After necropsy, only the small intestines of rabies-negative foxes were examined for tapeworms, and intestines were frozen at −80 °C for 7 days according to Ministry of Health guidelines (i.e. to avoid possible infection with *Echinococcus* spp.) prior to the examination via sedimentation and intestinal scraping (WHO/OIE Manual, 2001). Small intestines were cut into 5 pieces, opened and placed into water, and large food items removed. This was followed by a 30-min sedimentation period and by multiple washings, after which adult *Mesocestoides* spp. were collected and preserved in 70% ethanol.

Morphological identification of specimens

Cestode specimens subjected to morphological and molecular analyses were sampled from 10–15 foxes collected from each of the 5 localities in 2006, and 2–5 whole worms were selected from each fox on the basis of their completeness and quality of preservation.

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A subsample of these worms were also used for molecular analyses, in which case small portions of tissue were preserved in 95% EtOH, while the remainder of the worms were stored in 70% EtOH. Specimens used for morphometric analysis were rehydrated in a graded ethanol series and post-fixed for 24 h in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7·2). After washing for 1 h in PBS, specimens were stained overnight with Gill's haematoxylin (Sigma, US) diluted in tap water (pH = 6·0), dehydrated in a graded ethanol series, cleared in clove oil and mounted in Canada balsam. Identifications were based on comparison of 13 anatomical characters following the protocol of Skrjabin (1978) and specimens were compared with museum voucher specimens held in the Parasitic Worms Collection at the Natural History Museum, in London (NHM-PWC). In total, 35 specimens identified as *M. litteratus* and 20 identified as *M. lineatus* were measured using an Olympus BX 51 microscope and digital analysis imaging system. Significant differences in morphometrics between species were determined using a non-parametrical Kolmogorov-Smirnov test (P-level indicated in Table 2) implemented in Statistica ver. 6.0 (Tulsa, USA). Specimens are deposited in the NHM-PWC under accession numbers BMNH 2011.2.2.1-18 and BMNH 2011.2.2.19-20, and include those used for both morphological and molecular analyses (i.e. hologenophores, *sensu* Pleijel *et al*. 2008).

DNA isolation and PCR amplification

Ethanol in the tissue samples was replaced with Tris-EDTA buffer via soaking and total genomic DNA was extracted using a Qiagen DNeasy tissue kit following manufacturer's protocol, except that worms were incubated in proteinase K overnight and the gDNA eluted via 2 vols of 100 μl. Three μl of template gDNA were added to 25 μl PCR reactions using Ready-To-Go PCR beads (Amersham Pharmacia Biotech). The mitochondrial 12S and nuclear ITS-2 rDNA genes were amplified to provide direct comparison with the work of Padgett *et al*. (2005). Partial 12S sequences (~370 bps) were amplified and sequenced using the primers 60.for and 375.rev (Nickisch-Rosenegk *et al*. 1999), and complete ITS-2 sequences were amplified using primers NC-6 and NC-2 and sequenced together using primers NC-6-F1 and NC-2-R1 (Littlewood *et al*. 2008). In addition, partial sequences of the mitochondrial CO1 (~400 bps) and ND1 (~450 bps) genes were characterized using the primers Cyclo-cox1FA, Cyclo_16SRc and Cyclo_cox1Rb for CO1, and Cyclo_nad1F, Cyclo-trnNR and Cyclo_nad1Fb for ND1 (Littlewood *et al*. 2008). Fragments were amplified using the following thermocycling conditions: 94 °C/5 min denaturation hold; 40 cycles of 94 °C/30 sec, 52 °C/30 sec, 72 °C/1 min; and 72 °C/5 min extension hold. PCR products were visualized on a 1·5% agarose gel and either gel-excised using a Qiagen QIAquick Gel Extraction Kit or purified directly using a Qiagen PCR Purification Kit, and then cycle-sequenced from both strands using ABI BigDye™ chemistry, alcohol precipitated and run on an ABI automated Sanger-based sequencer.

Sequence alignment and phylogenetic analyses

Amplification was successful in samples of 38 of the isolates (BA = 2, DK = 8, KE = 13, PO = 6, ZV = 7) representing the majority of specimens used for morphometric analysis, although not all samples yielded results for all genes. Thus 31, 25 and 26 *Mesocestoides* sequences were characterized for 12S, CO1 and ND1 partitions, respectively, plus 2 additional CO1 and ND1 sequences of the cyclophyllidean *Taenia taeniaformis* from red foxes in Košice and Zvolen included for outgroup comparison. Sequences were assembled and edited manually using Sequencher ver. 4.6 (GeneCodes Corporation). Regions corresponding to the PCR primers were removed prior to analysis. All sequence identities were verified using the Basic Local Alignment Search Tool (BLAST) (McGinnis and Madden, 2004) (www.ncbi.nih.gov/BLAST/). All sequences are deposited in GenBank under accession numbers JF 268553-581 (12S), JF 268498-525 (CO1) and JF 268526-552 (ND1).

The composition and number of additional *Mesocestoides* and outgroup sequences included varied, based on the availability of relevant sequences for the different gene partitions. For the 12S alignment, the comparatively large number of North American isolates from Padgett *et al*. (2005) were included along with several other available sequences, whereas only additional *Mesocestoides* sequences were available for CO1 (AB033413 and EU665469), and 1 for ND1 (EU665480). All data partitions were rooted using cyclophyllidean taxa (see Fig. 3 for sequence Accession numbers). Sequences were aligned initially with ClustalX v 2.0 (Thompson *et al*. 1997) using default parameter settings (i.e. gap opening and gap extension fixed at 10 and 0·2 respectively. Alignments were further improved by eye using MacClade ver. 4.08 (Maddison and Maddison, 2000), with the protein-coding CO1 and ND1 sequences aligned according to codon positions, with the genetic code set to 'flatworm mtDNA' (Nakao *et al*. 2000). Alignments were truncated to remove leading and trailing gaps, as well as regions that contained alignment gaps in the majority of taxa.

Phylogenetic trees were constructed using maximum parsimony, maximum likelihood and Bayesian inference. Maximum parsimony was performed using PAUP* ver. 4.0b (Swofford, 2003). A heuristic search (1000 replicates), random-sequence addition

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Table 1. Prevalence of *Mesocestoides* spp. in red foxes from six administrative districts of the Slovak Republic from 2001–2006

	2001		2002		2003		2004		2005		2006		2001–2006	
Locality†	N	P (%)	N	P (%)	N	P (%)	N	P (%)	N	P (%)	N	P (%)	N	P (%)
BA	21	76·2	46	32·6	76	29·0	–	–	20	35·0	210	15·2	373	24·7
NR	62	85·5	131	51·9	65	49·2	–	–	16	25·0	19	31·6	293	55·6
ZV	9	66·7	161	38·5	203	32·5	–	–	80	32·8	130	46·9	583	37·9
DK	39	74·4	52	55·8	93	51·6	87	39·1	98	40·8	138	38·4	507	46·0
PO	49	77·6	133	57·1	220	37·7	142	47·9	42	33·8	72	30·6	658	57·0
KE	70	61·4	119	47·1	225	32·9	119	70·6	36	25·0	174	26·4	743	42·0
Total	**250**	**74·0**	**642**	**47·7**	**882**	**36·8**	**348**	**53·5**	**292**	**34·3**	**743**	**29·6**	**3157**	**41·9**

† BA, Bratislava; NR, Nitra; ZV, Zvolen; DK, Dolný Kubín; PO, Prešov; KE, Košice.

and tree bisection and reconnection (TBR) options were used, with all characters unweighted and unordered and gaps treated as missing data. To determine branch support, 1000 bootstrap replicates were generated. Maximum likelihood analysis was performed using PhyML v2.4.4 (Guindon and Gascuel, 2003) with the best-fit model of nucleotide substitution selected using MrModelTest ver. 2 (Nylander, 2004): GTR+G for 12S rDNA, HKY+G for CO1 and GTR+I+G for ND1. Analyses of CO1 and ND1 protein translations employed the WAG amino acid substitution model (Whelan and Goldman, 2001).

Bayesian analysis was performed with MrBayes v3.2 (Ronquist and Huelsenbeck, 2003) using the substitution models selected above and default prior probabilities set to be estimated (Dirichlet (1,1,1,1)). Chain length was set to 1 000 000 generations sampling every 100th generation. Two simultaneous independent runs were undertaken for each dataset starting from different random trees with the 'burn-in' set to 10% (100 000 generations). To ensure convergence, parameter estimates were examined using Tracer ver. 1.4 (part of the BEAST package; Drummond and Rambaut, 2007). Perl scripts were written to automate the majority of the phylogenetic analyses (available on request from AOC) and Geneious ver. 5.0 (Drummond *et al*. 2010) was used for both analysis and visualization of the data.

RESULTS

Prevalence of Mesocestoides *spp. in red foxes in Slovakia*

Of the 3157 foxes examined over a 6-year period, 1322 harboured infections with *Mesocestoides* spp. (41·9%), and prevalence fluctuated considerably among localities and years (Table 1). In the locality near Bratislava encompassing a largely urban area, the mean prevalence was 24·7%. For the 5 remaining administrative regions that were mostly forested, a higher density of foxes was associated with higher rates of infection, with the highest overall prevalence of 57% recorded from the Prešov district adjacent to the Tatra National Park in the High Tatras mountains. Among the years of study, the highest infection rate (74·0%) was seen in all 6 regions in 2001 and the lowest (29·6%) in 2006. Intensities of infection ranged from 10 to several hundred worms, but the inability to make positive identifications in the field meant that we could not record intensity data for individual *Mesocestoides* species.

Two species of *Mesocestoides* were identified in the samples that were consistent with the descriptions of *M. litteratus* (Batsch, 1786) and *M. lineatus* (Goeze, 1782) and to voucher specimens of these species (BMNH 6.14.25.44 and BMNH 6.14.25.45, respectively, *Ex. Vulpes vulpes* from Iraq) deposited in the NHM-PWC. Of the two species, *M. litteratus* was far more prevalent and widespread than *M. lineatus*, which was present only in the regions of Košice, Prešov, Dolný Kubín and Zvolen (Fig. 1), where it had a prevalence of only 7%. With the exception of 1 fox from Dolný Kubín that harboured a mixed infection, all other foxes hosted infections with single species.

Morphometric diagnosis of M. litteratus *and* M. lineatus

No significant difference was seen in the total length of gravid worms, which varied considerably in both species (4–18 cm; Table 2). Scolex morphology was similar in appearance and size, consisting of 4 simple suckers and lacking a rostellum. No significant difference was found either among scolex characters or for the width and length of mature proglottids. Specimens identified as *M. litteratus* (Fig. 2A–C) had an elongate cirrus sac, the cirrus was muscular, straight or formed 1 or 2 small curves. In contrast, specimens of *M. lineatus* (Fig. 2D–F) had a rounder cirrus sac with a thinner, longer cirrus forming a few coils. The cirrus sac was significantly longer in *M. litteratus* than *M. lineatus* ($P < 0{\cdot}01$). Localization

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Fig. 1. Collection locations of red foxes (*Vulpes vulpes*) in the Slovak Republic and distribution of *Mesocestoides litteratus* (open circles) and *Mesocestoides lineatus* (black circles) species according to the area of host origin.

and size of ovaries was different in both species. They were localized at some distance from the posterior margin of proglottids in *M. litteratus* and were elongate in shape. In the mature proglottids of *M. lineatus*, ovaries were round to oval and were localized adjacent to the posterior end of the proglottids. There were significant differences in the width ($P<0{\cdot}01$) and length ($P<0{\cdot}05$) of the ovaries between species. Vittelaria were bi-lobed and ventral, partially covered by the ovaries. A significant difference ($P<0{\cdot}01$) was found in the number of testes, which were less numerous in *M. lineatus* (29 ± 3) than in *M. litteratus* (56 ± 5). Testes formed 2 lateral fields in mature proglottids, which merged in the middle, posterior and anterior to the female system in *M. litteratus* (Fig. 2A), but only in the anterior part in *M. lineatus* (Fig. 2D). Pre-gravid segments of *M. lineatus* (Fig. 2E) were significantly shorter ($P<0{\cdot}01$) than segments of *M. litteratus* (Fig. 2B), and contained the cirrus sac, tube-like uterus and parauterine organ. In gravid proglottids of both species the uterus was either rudimentary or absent. Eggs were accumulated in the fully developed parauterine organ, which was oval in *M. litteratus* (Fig. 2C), more round and smaller in *M. lineatus* (Fig. 2F) ($P< 0{\cdot}01$) and the size of eggs was not significantly different between the species.

Phylogenetic analyses

Characterization of ITS-2 showed the presence of more than 1 copy of the gene in the Slovakian samples, resulting in sequence trace files confounded by multiple signals. The presence of 2–3 intraspecific haplotypes per sample was identified by cloning several of the PCR amplicons using a TOPO-TA kit (Invitrogen), and for this reason, no further analysis of the ITS-2 data was conducted.

Analyses of the 12S, CO1 and ND1 data partitions strongly supported the existence of independent clades of *M. litteratus* and *M. lineatus*, consistent with their diagnosis on morphological features described above. Estimated divergence between *Mesocestoides* species was 13% in ND1, 9–15% in CO1, and 16–18% in 12S. By comparison, intraspecific divergence within *M. lineatus* and *M. litteratus* was less than 1·5%. Phylogenetic analyses (Fig. 3) showed that the European *M. lineatus* and *M. litteratus* isolates are not sister species, and that *M. litteratus* groups are within the clade of North American isolates (Fig. 3), as the sister group to 'Clade C' as defined by Padgett *et al*. (2005). Substructuring within the *M. litteratus* clade is poorly resolved by our data, albeit some consistencies among the data partitions (e.g. grouping of isolates KE903, ZV889 and ZV903) suggest a degree of non-random sorting of the mitochondrial genes. At the amino acid level, however, analyses of CO1 and ND1 show separation of the *Mesocestoides* spp., but provide no support for the subspecific structure of the *M. litteratus* isolates (trees not shown). Phylogenetic estimates were the same regardless of the method of analysis used.

DISCUSSION

Results from 6 years of monitoring of red foxes in Slovakia show that the prevalence of *Mesocestoides* infections can be as high as 85% in regions of dense forestation, and that there is a high (42%) overall prevalence in foxes throughout the country. Mountainous regions in the north (Dolný Kubín) and northeast (Prešov) showed the highest

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Table 2. Comparison of morphometric measurements of adult *Mesocestoides litteratus* and *M. lineatus* from red foxes in the Slovak Republic

(All measurements are in μm except where noted and are given in width × length and the mean ± S.D. (range).)

	M. litteratus	*M. lineatus*	Sig. diff.
No. of worms	35	20	
Length (cm)	13 ± 5 (4.5–8.4)	9 ± 4 (3.6–13.6)	–
Mature segments	715 ± 94 (520–845) × 659 ± 141 (480–791)	744 ± 55 (470–913) × 538 ± 61 (415–618)	– –
Pre-gravid segments	921 ± 185 (727–1,275) × 1,725 ± 203 (1,454–2,225)	846 ± 49 (788–1,015) × 1038 ± 74 (876–1,152)	– P<0.01
Gravid segments	1,129 ± 69 (1,090–1,277) × 2,457 ± 267 (2,792–3,360)	1,121 ± 106 (974–1,255) × 1,918 ± 159 (1,767–2,120)	– P<0.01
Scolex	549 ± 47 (485–620) × 437 ± 48 (368–510)	532 ± 37 (477–580) × 402 ± 19 (310–440)	– –
Suckers	190 ± 14 (167–220) × 200 ± 16 (185–235)	182 ± 11 (155–195) × 196 ± 23 (187–259)	– –
No. of testes	56 ± 5 (50–75)	29 ± 3 (25–38)	P<0.01
Testes	26 ± 4 (21–30) × 45 ± 7 (30–58)	41 ± 5 (33–47) × 52 ± 5 (47–66)	P<0.01 –
Cirrus sac	57 ± 8 (40–70) × 198 ± 19 (167–216)	87 ± 8 (77–99) × 111 ± 48 (82–147)	P<0.01 P<0.01
Ovaries	43 ± 12 (28–64) × 144 ± 27 (98–190)	77 ± 9 (67 – 95) × 110 ± 14 (94–133)	P<0.01 P<0.05
Vitellaria	54 ± 8 (40– 68) × 112 ± 13 (98–135)	66 ± 12 (48–85) × 95 ± 9 (78–108)	– –
Parauterine organ	400 ± 51 (320–467) × 551 ± 79 (424–680)	287 ± 21 (256–340) × 416 ± 57 (330–478)	P<0.01 P<0.01
Eggs	29 ± 3 (25–35)	30 ± 5 (22–37)	–

prevalences, and the lowest were found in more urban areas in the west, near Bratislava. Annual fluctuations in prevalence were likely due to the climatic factors that alter the population densities of foxes and intermediate host populations. Miterpáková *et al*. (2003, 2006) examined the tapeworm *Echinococcus multilocularis* in red foxes in Slovakia and found significant correlations between the prevalence, type of habitat and climatic conditions. Their work showed that the prevalence of *E. multilocularis* ranged from 25% to 33% and that co-infections with *Mesocestoides* spp. were common.

We report, for the first time, the occurrence of *M. lineatus* in the territory of Slovakia and show that it can be differentiated from *M. litteratus* using both morphological and molecular data. *M. lineatus* has previously been reported from foxes in countries situated to the north and northwest of Slovakia, i.e. Poland (Okulewicz *et al*. 2005), Denmark (Willingham *et al*. 1996), Greenland (Kapel and Nansen, 1996), France (Pétavy *et al*. 1990), and to the south of Slovakia, i.e. Hungary (Gubány and Eszterbauer, 1998). Although its prevalence in Slovakia was low (5–7%), distribution was widespread in the north and northeast of the country. In contrast, *M. litteratus* was the dominant species found in all 6 regions and accounted for most of the high prevalence seen in the country, just as it was in the countries situated to the south and west of Slovakia (Literák *et al*. 2006).

There have long been, and there still remain, uncertainties surrounding the taxonomy of *Mesocestoides* species, and it is clear that diagnosing specific lineages in the genus cannot be based on characters of the scolex and other features that show high levels of variation. However, our results corroborate older taxonomic studies (e.g. Skrjabin, 1978; Jančev, 1986) that show that *M. lineatus* and *M. litteraus* can be differentiated on the basis of proglottide morphology (i.e. shape of the cirrus sac, length of cirrus, number of testes and position of ovaries). Our measurements of the Slovakian isolates were also regularly within the range of those reported by Loos-Frank (1980*b*), Jancev (1986) and Gubány and Eszterbauer (1998), based on specimens from Germany, Bulgaria and Hungary, respectively. Although Padgett *et al*. (2005) reported that the 3 main clades of North American isolates recovered by their 12S sequences could not be distinguished via morphometric analysis, their study compared only the sizes of the scolex, suckers and parauterine organ, all of which showed tremendous ranges. Morphological diagnosis of European *Mesocestoides* suggests that analyses of proglottide morphology may have yielded character differences among the 3 clades not found by Padgett *et al*. (2005).

Molecular data partitions strongly supported the existence of individual clades of *M. litteratus* and *M. lineatus*, and showed that the two species do not share a most recent common ancestor. Instead,

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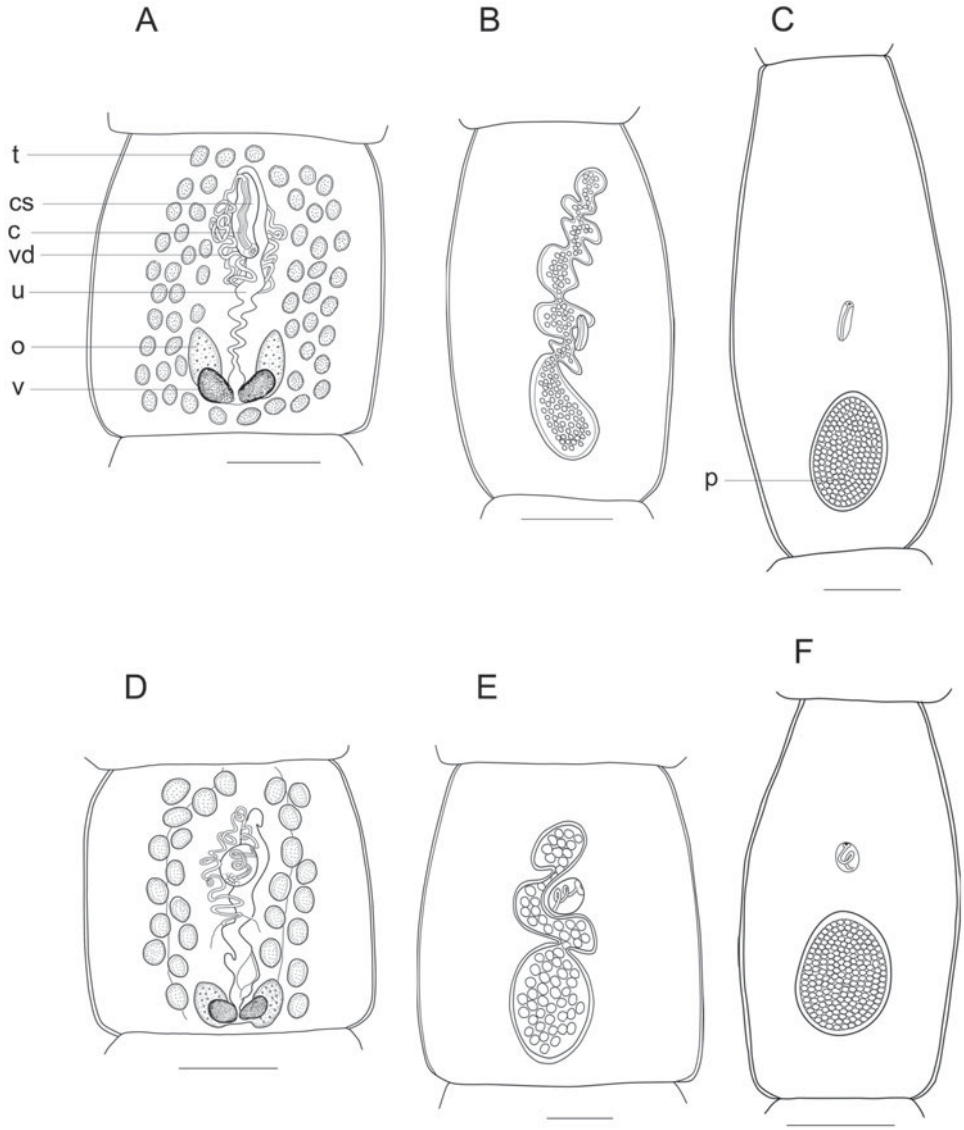

Fig. 2. Diagnostic line drawings of the proglottide morphology of *Mesocestoides litteratus* (A–C) and *M. lineatus* (D–F). A, D: mature proglottid; B, E: pre-gravid; C, F: gravid. cs, cirrus sac; c, cirrus; o, ovaries; t, testes; u, uterus; v, vitellaria, vd, vas deferens. Scale bars: A, E, D = 200 μm; B, C, F = 500 μm.

M. litteratus was found to be closer to the North American isolates examined by Padgett *et al.* (2005) and formed a sister lineage to an as yet unidentified species of *Mesocestoides* infecting foxes and dogs on the west coast of the USA (i.e. clade 'C'). *M. lineatus* was positioned outside this large group of European and North American isolates, and a better understanding of its phylogenetic affinities clearly requires broader sampling of species in the genus. Within the *M. litteratus* clade, genetic divergence was similar to that seen within the North American 'species' clades. Six haplotypes were present in the 12S rDNA, but only 2 of these subgroupings were also supported by the CO1 and ND1 data. These haplotypes were shared among isolates from Bratislava and Dolný Kubín in one instance, and from Košice and Zvolen in the other, but like the other subgroupings produced by the various data partitions, the individual haplotypes did not appear to reflect geographical separation, but rather showed that most haplotypes were widespread within Slovakia.

Some discussion over recent decades has evolved around the taxonomic status *M. leptothylacus* described from foxes from southwest Germany (Loos-Frank, 1980*b*; Loos-Frank and Zehle, 1982). According to Loos-Frank (1980*b*) the species is most similar to *M. erschovi*, but Priemer *et al.* (1983) advocated conspecificity of *M. leptothylacus* and *M. litteratus*. In our analyses, it is clear that the sequence of *M. leptothylacus* is part of the *M. litteratus* clade and differs genetically by no more than 2% from the other Slovakian haplotypes. By comparison with interspecific divergences in *Mesocestoides* of 8–14%, we consider that the divergence in this isolate most likely represents geographical variability within European *M. litteratus* and thus does not support species status of *M. leptothylactus*. However, our analyses also show that a number of published

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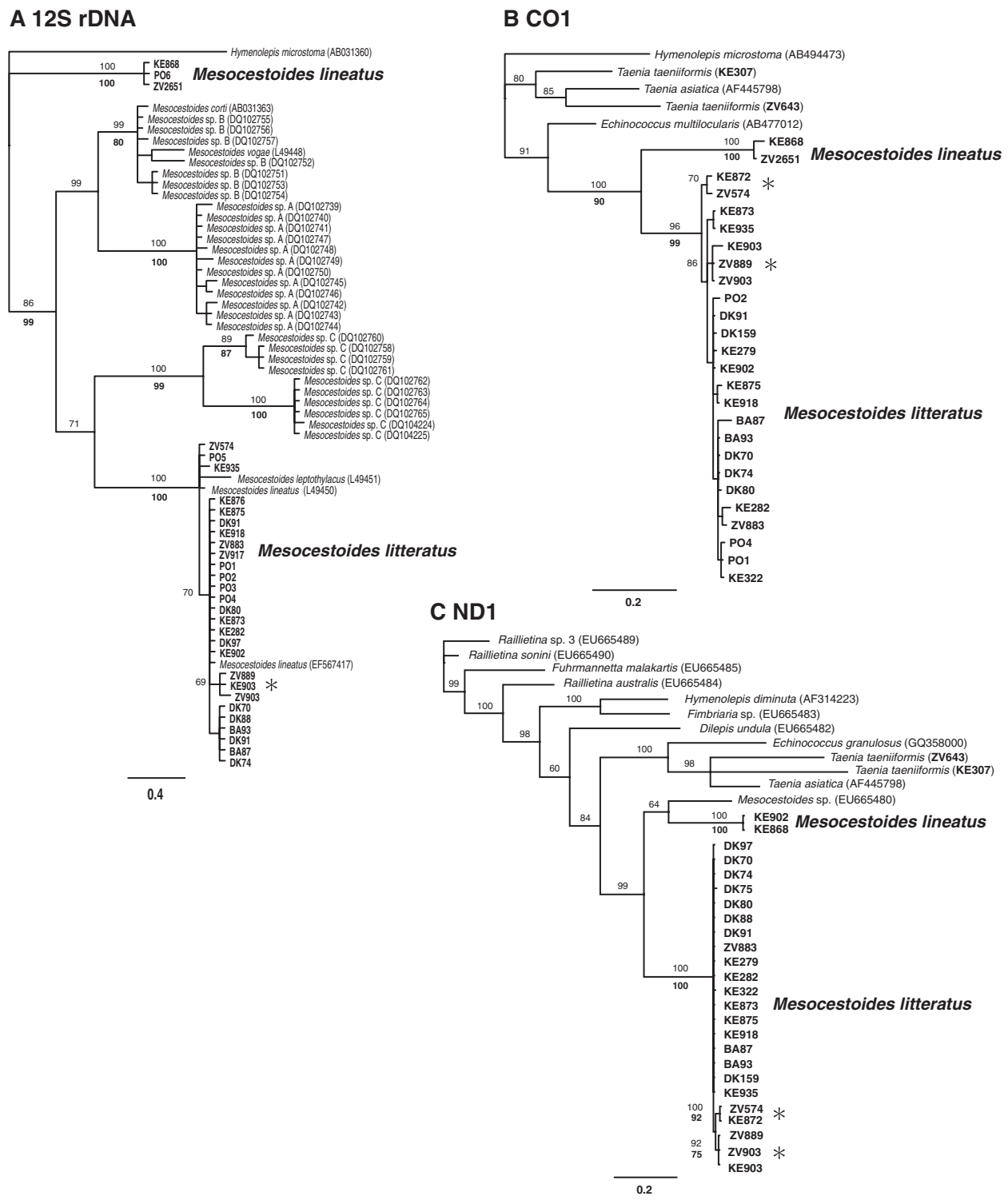

Fig. 3. Bayesian inference analyses of the molecular data partitions. Nodal support shows posterior probabilities (above) and bootstrap support ⩾50% (below). Asterisks indicate subclades of *Mesocestoides litteratus* discussed in the text.

sequences found within the *M. litteratus* clade have been previously misidentified (e.g. as *M. lineatus*: EF567417, L49450) and it is not clear if an isolate identified as *M. leptothylacus* by the authors Nikish-Rosengeek *et al.* (1999) was in fact consistent with the morphological diagnosis of that species.

The presence of independent copies of ITS-2 rDNA (i.e. paralogues) confounded our attempts to characterize these loci in the Slovakian isolates of *Mesocestoides* and compare them with those of the North American species, in which the existence of multiple paralogues was not reported (Padgett *et al.* 2005). The presence of multiple, independent rDNA arrays in the tapeworm genome were recently demonstrated via chromosomal *in situ* hybridization by Králová-Hromadová *et al.* (2010) in the

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caryophyllidean *Atractolytocestus*. Their study is the first to provide physical evidence of multiple arrays in the genome, but reports of intraspecific variation in nuclear ITS sequences in other tapeworms groups show that it is frequently observed (Olson and Tkach, 2005), giving the ITS rDNA regions limited diagnostic and phylogenetic utility in these parasites.

Our work shows that the encroachment of red foxes into urban areas in central Europe brings with it a high rate of *Mesocestoides* infection. Concurrent infections with *Echinococcus multilocularis* and the fact that these tapeworms can be hosted by domestic animals means that the risk to human populations merits the continued control and monitoring of fox populations in the region. Comparable molecular characterizations of isolates taken from both clinical and field settings is essential for a better understanding of species boundaries and host associations in *Mesocestoides* and for assessing the impact of the parasites on human health.

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