Phylogenetic analysis of partial mitochondrial cytochrome oxidase c subunit I and large ribosomal RNA sequences and nuclear internal transcribed spacer I sequences from species of Cyathostominae and Strongylinae (Nematoda, Order Strongylida), parasites of the horse

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SUMMARY

Three nucleotide data sets, one nuclear (ITS-2) and two mitochondrial (COI and l-rRNA), have been investigated in order to determine relationships among species of Strongylinae and Cyathostominae, intestinal parasites of the horse.† The data exhibited a strong mutational bias towards A and T and in the COI gene, silent sites appeared to saturate rapidly partly due to this substitution bias. Thus, the COI gene was found to be less phylogenetically informative than the l-rRNA and ITS-2 genes. Combined analysis of the l-rRNA and ITS-2 genes supported a monophyletic clade of the cyathostomes with *Tridentoinfundibulum gobi*, which had previously been classified as a nematode of 'uncertain origin'. The Strongylinae grouped consistently outside the clade containing the cyathostomes and *T. gobi*. Molecular analysis failed to provide strong evidence for the separation of cyathostomes into classical genera, as previously defined by morphological classification.

Key words: horse strongyles, Strongylinae, Cyathostominae, molecular phylogeny, substitution bias, combined analysis.

INTRODUCTION

The large intestinal parasites of the order Strongylida are of major veterinary importance in the horse. Within the order Strongylida, the Strongylinae or 'large strongyles' and the Cyathostominae or 'small strongyles' are the parasitic subfamilies which cause significant diseases in equids. Previously, *Strongylus* spp., members of the Family Strongylinae were considered the most pathogenic parasites, but increased use of modern, efficient anthelmintics in parasite control programmes has resulted in effective reduction in prevalence and incidence of disease due to these parasites. Much attention has been focussed on the Cyathostominae in recent years due to increased recognition of resistance to commonly used anthelmintics such as benzimidazoles (Herd, 1990; Herd & Coles, 1995) and pyrantel (Chapman *et al*. 1996), making effective control of these parasites very difficult. Cyathostomes are now recognized as the most important and pathologically significant parasite of the horse. Infection with these parasites is a source of considerable economic loss to the horse industry. In order to understand these problems and evaluate possible control strategies, it is necessary to identify the species involved. A total of 51 species of small strongyles and their generic groupings have been listed by Lichtenfels *et al*. (1998), and this classification incorporates those of the most recent morphological descriptions of these nematodes (Hartwich, 1986; Lichtenfels, 1975, 1980). Based on surveys of adult parasites collected at post-mortem, it has been observed that the same 5–10 species of cyathostomes are prevalent in domestic equine populations world-wide (Mfitilodze & Hutchinson, 1990; Bucknell, Gasser & Beveridge, 1995; Gawor, 1995). While the pathogenic effects of cyathostome infestation have been documented (Love, 1992; Love, Mair & Hillyer, 1992), there is a relative paucity of information on the evolutionary or systematic relationships of these parasites to each other and to members of the Strongylinae.

There is also disagreement in the current literature as to the phylogenetic relationships between the two

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subfamilies of the strongylid nematodes of horses. Current phylogenetic classifications are based on morphological features (of which there are relatively few in nematodes) and which may be problematic when used for phylogenetic inference (Hillis, Moritz & Mable, 1996). Lichtenfels (1980, 1987) and Durette-Desset, Beveridge & Spratt (1994) have raised the viewpoint that the arbitrary separation of the Strongylinae and Cyathostominae on the basis of size and shape of the buccal capsule should be re-examined to determine whether the two families are, indeed, natural groups. These authors considered the genera of equine strongyles with large subglobular buccal capsules (Strongylinae) to be ancestral to those with small cylindrical buccal capsules (Cyathostominae). Dvojnos (1982), in contrast, considered the genera of equine strongylids to be monophyletic but considered the genera with cylindrical buccal capsules to be primitive. He suggested that genera with large buccal capsules had evolved from genera with small buccal capsules in several lines within the group.

The objective of this study was to determine the evolutionary relationships of the Cyathostominae to each other and to members of the subfamily Strongylinae using data derived from DNA sequences. Two mitochondrial gene sequences, cytochrome oxidase I (COI) and the l-rRNA subunit (l-rRNA), and one nuclear sequence, the second internal transcribed spacer of the rDNA array (ITS-2) were used to reconstruct phylogeny. These genes were chosen based on their usefulness in reconstructing phylogeny in other parasite taxa. The COI gene has been used to study evolutionary relationships among recently diverged/rapidly evolving taxa and also to resolve deep branch phylogenies in which multiple substitutions are a critical problem (Bowles & McManus, 1993; Kumazawa & Nishida, 1993; Sukhdeo *et al*. 1997; Morgan & Blair, 1998). The l-rRNA gene has been used to reconstruct phylogenetic relationships at the subgeneric level (DeSalle *et al*. 1992; Vogler & DeSalle, 1993) as well as at deeper phylogenetic levels (Flook & Rowell, 1997). The ITS-2 has been used to identify equine strongylid nematodes to species level (Campbell, Gasser & Chilton, 1995; Gasser *et al*. 1996), to distinguish cryptic species (Chilton, Gasser & Beveridge, 1995; Hung *et al*. 1999*a*) and to reconstruct phylogeny (Chilton, Gasser & Beveridge, 1997; Hung *et al*. 1999*b*, 2000).

MATERIALS AND METHODS

Sources and processing of parasite isolates and DNA purification

Adult parasites were collected from the large intestinal contents of 4 ponies and 1 horse, humanely destroyed for reasons other than parasite infestation. These animals were of Scottish origin. The parasite species used and their sources are listed in Table 1. We also used genomic DNA from individuals of a selection of cyathostome species and 3 species of *Strongylus* from the UK, USA and Australia, in order to examine population variation.

The parasites collected from the indigenous population were washed once in sterile phosphate-buffered saline, pH 7·2 followed by 2 washes with sterile Hanks Buffered Salt Solution (Gibco-BRL Ltd, Scotland) containing penicillin (10 IU/ml) and gentamicin (10 mg/ml). The bodies were cryopreserved, the corresponding heads were excised and mounted on glass slides using a 5% phenol–alcohol–glycerine solution, as described by Lichtenfels *et al*. (1998). Individual parasite heads were identified according to the key of Lichtenfels (1975). The names for the Cyathostominae followed those published by Lichtenfels *et al*. (1998). DNA was isolated from individual parasites by sodium dodecyl-sulphate/proteinase K treatment for 16 h followed by phenol–chloroform/isoamyl alcohol (25:24:1 ratio) extraction.

Primer design and PCR amplification

The cytochrome oxidase I (COI) primers were based on evolutionarily conserved regions of the COI sequence for the trematode, *Fasciola hepatica* (Garey & Wolstenholme, 1989), and subsequently aligned with sequences of 2 Secernentean worms, *Ascaris suum* and *Caenorhabditis elegans*. Two single base pair mismatches were introduced at base positions 9 and 18 in the *F. hepatica* COI forward primer sequence as shown: 5′ TTTTTTGGTCATCCTG-AACTTTAT 3′, in order to make it more similar to the *A. suum* and *C. elegans* sequences. The sequence of the COI reverse primer was: 5′ TAAAGAAAG-AACATAATGAAATG 3′. These primers were designed to amplify a 393 bp portion of the COI gene.

The large ribosomal (l-rRNA) DNA primers were designed to amplify the terminal portion (last 70 bp) of the 3′ end of the COII gene and a portion of the 5′ end of the adjacent l-rRNA. These degenerate primers were a gift from Dr T. J. C. Anderson, University of Oxford. The primers were designed based on an alignment of the mitochondrial DNA sequences of nematodes from both classes of the phylum Nematoda (an Adenophorean nematode, *Romanomermis culicivorax*) and 3 Secernentean nematodes, *C. elegans*, *A. suum* and *Meloidogyne incognita*. The primer sequences were: forward primer; 5′ GGTCAATGTTCNGARATTTGTR-G 3′ and reverse primer 5′ ACYMMTCACGCTA-ARAASTGTRG 3′. The ITS-2 primers NC1 and NC2 were designed as described by Hung *et al*. (1997), from the conserved portions of the flanking regions of the ITS-2 sequence of *C. elegans*. Oligonucleotides were synthesized by Perkin Elmer-

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Table 1. Species and sources of parasites sequenced in this study.

(All species are members of the Subfamily Cyathominae (a) or Strongylinae (b) *T. gobi* is an nematode of ''uncertain classification'' (Lichtenfels, 1975)

Species	Source
Coronocyclus coronatus (*a*)	Scotland
Craterostomum acuticaudatum (*a*)	Scotland
Cylicocyclus ashworthi (*a*)	Scotland/Australia
Cylicocyclus insigne (*a*)	Scotland/Australia
Cylicocyclus nassatus (*a*)	Scotland/Australia
Cyathostomum pateratum (*a*)	Scotland
Cylicostephanus calicatus (*a*)	Scotland
Cylicostephanus goldi (*a*)	Scotland
Cylicostephanus longibursatus (*a*)	Scotland
Strongylus edentatus (*b*)	Australia/USA
Strongylus equinus (*b*)	Australia/USA
Strongylus vulgaris (*b*)	Australia/USA
Tridentoinfundibulum gobi	Scotland
Triodontophorus serratus (*b*)	Scotland

ABI Ltd (Warrington, Cheshire, UK). PCR reaction mixtures (50 μl) consisted of 3 mM $MgCl_2$, 200 μm each dNTP, 0·5 μM each primer and 1 U of AmpliTaq DNA polymerase (Perkin-Elmer, ABI Ltd). DNA from each individual was amplified in duplicate. Genomic DNA from each individual worm was diluted 1:5 and 1:10, and 1 μl of each dilution was used as template in the PCR. In each PCR experiment, positive (*A. suum* genomic DNA for the COI and l-rRNA PCR and *T. gobi* DNA for the ITS-2 PCR) and negative (no DNA) control reactions were included. Host DNA was used as a control to ensure the specificity of each set of primers used in the PCR experiments.

The amplification programme for the COI PCR consisted of an initial denaturation step of 94 °C for 4 min. Thermal cycling was over 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. This was followed by a final extension at 72 °C for 10 min. For the l-rRNA PCR, following an initial denaturation step of 94 °C for 4 min, thermal cycling was over 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. This was followed by a final extension at 72 °C for 10 min. The thermal cycling conditions for the ITS-2 PCR were as described by Hung *et al*. (1997). PCR products were detected on 1 × TAE, 1 % agarose gels stained with ethidium bromide. For the negative control samples, at least 50 % of the sample was examined on the gel.

DNA sequencing

Two PCR products per worm were sequenced in both directions using an ABI Prism™ Dye Terminator Cycle Sequencing kit (ABI-Perkin-Elmer Co.) on an ABI 377 machine according to the manufacturer's instructions. Sequences were analysed using ABI-Perkin Elmer sequence analysis software (Version 2.1.1).

Sequence alignment

The COI sequences were aligned using Pileup in Wisconsin GCG, Version 9. Clustal X (Thompson *et al*. 1997) was used for alignment of the ITS-2 sequences. For the ITS-2 gene, the 5′ and 3′ ends of the gene were determined by comparison with the sequences from other species of strongylid nematodes (Campbell, Gasser & Chilton, 1995; Hung *et al*. 1997). Alignment of the l-rRNA sequences was accomplished using both secondary structure and sequence data. Clustal X (Thompson *et al*. 1997) was used to align the sequence data. The l-rRNA sequences were aligned at the 5′ and 3′ ends of the gene by comparison with the sequences from *A. suum* and *C. elegans* (Okimoto *et al*. 1992). Secondary structure alignments for the l-rRNA sequences were performed using the programme GlasgalV (R. H. Wilson, University of Glasgow, personal communication, 1998). The secondary structure alignments were viewed in relation to the hypothetical 3-dimensional structure of *C. elegans* l-rRNA (Gutell, Schnare & Gray, 1993; Okimoto, MacFarlane & Wolstenholme, 1994).

Analysis of substitutional bias in mitochondrial and nuclear sequences

In order to assess the nature of the substitution patterns, all transitions, AT transversions and all other types of transversions were determined in PAUP*4.0d64 and values obtained were plotted against pairwise distances calculated using ML under a GTR model estimated from the data for each sequence/region. For the COI gene sequence, substitution types for each codon position were plotted individually as well as for all codon positions combined. The transition to transversion ratio (TS:TV) for each gene was also determined in PAUP*4.0d64 and plotted against pairwise distances using the GTR model as described above.

Phylogenetic analysis

Phylogenetic analysis was performed using PAUP* 4.0d64 (Swofford, 1998). Each gene was analysed separately in the first instance, using Neighbour-Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) methods. Alignment gaps were treated as missing character states for the analyses. A minimum of 4–6 individuals of each species were initially sequenced for the COI gene (the first gene to be investigated) and were found to cluster with other members of their species group on NJ analysis. Intraspecific variation ranged from 1 to 6 %. Where available, an Australian isolate of each

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species was included in this preliminary analysis (to examine the influence of geographical variation), and was found to cluster within the appropriate species group. Subsequently, 1 individual from each of these species groups (randomly chosen) was used for further phylogenetic analysis in the l-rRNA and ITS-2 sequences. Distance correction was applied using ML under a General Time Reversible model (GTR) with starting parameters estimated from the data. The GTR model (Lanave *et al*. 1984) used under ML, accounts for biased nucleotide composition and allows the user to input the expected rates of substitution at different sites.

RESULTS

Characteristics of amplicons and sequences

All COI PCR products obtained were 393 bp, while ITS-2 PCR products ranged from 217 bp to 334 bp. The sizes of the ITS-2 PCR products obtained were in overall agreement with those reported by Campbell *et al*. (1995) and Hung *et al*. (1997, 1999*b*). The l-rRNA PCR products ranged in size from 513 to 529 bp. The A+T composition for the COI gene was 67%, for the ITS-2 gene it was 60% and for the l-rRNA gene it was highest at 82%.

Analysis of substitution bias in mitochondrial and nuclear gene sequences

Figure 1A, C and E show all transitions, A↔T transversions and all other transversions plotted against pairwise distances calculated under ML for each gene. The nematode mitochondrial genome is highly biased in favour of adenine (A) and thymine (T) nucleotides (Okimoto *et al*. 1992). In the COI gene, the A+T composition of the first (23%) and second position (47%) sites was lower than that of third position sites (68%). Blouin *et al*. (1998) also reported a similar finding in their analysis of the mtND4 gene. In each sequence/region, A↔T transversions were most numerous, reflecting the extreme A+T bias in the data, and all other transversions were observed to occur rarely by comparison. Figure 1B suggests evidence for saturation in the COI gene; the least different sequences show a large number of transitions for every observed transversion, the more distantly related pairs showing a much smaller ratio. The transition substitution pattern observed at the third codon position in the COI gene began to become more linear at pairwise distances greater than 20%, suggesting that above this distance, saturation would occur (data not shown). Figure 1C shows, for the ITS-2, a strong linear relationship of transitions versus distances for the most similar sequences only. More divergent sequences show a wide range of pairwise transition differences. In contrast, the transversions continue to accrue in a presumably time-dependent fashion. Figure 1E shows that for the l-rRNA, A↔T transversions rapidly outnumbered transitions with increasing genetic distance. The TS:TV ratio (Fig 1B, D and F) for all sequences/regions showed the expected decrease with increasing genetic distance (Morgan & Blair, 1998; Blouin *et al*. 1998).

Phylogenetic analysis of COI sequences

Phylogenetic analysis of the COI gene by NJ, MP and ML, based on sequence data, produced trees with clades that were poorly supported on bootstrap analysis, apart from the grouping of sister taxa such as *S. equinus* with *S. edentatus* and *C. longibursatus* with *C. goldi* (data not shown). Pairwise distances between taxa in Strongyloidea (calculated under ML using a GTR model estimated from the data) ranged from 11 to 20%. At this level of pairwise difference, it has been suggested that saturation occurs at the third base position (Meyer, 1994). Other authors have also reported third-position sites in nematode mtDNA protein-coding genes to be saturated and uninformative in higher level phylogenetic reconstruction (Blouin *et al*. 1998; Zarlenga *et al*. 1998). Thus, the third codon position was excluded from further analysis. Unfortunately, no variation at the first and second codon position resulted in an even poorer phylogenetic signal. Despite differences in the primary DNA sequences, which were in all cases, at the third codon position, all taxa translated highly similar amino acid sequences such that there was insufficient phylogenetic signal to support phylogenetic groupings when amino acid data were analysed by NJ or MP.

Phylogenetic analysis of the l-rRNA and ITS-2 sequences

In contrast to the results obtained with the COI gene sequences, analysis of the l-rRNA and ITS-2 gene sequences individually by NJ, MP and ML produced phylogenetic trees with overall similar topology which were well supported on bootstrap analysis, irrespective of the method used (data not shown). There were some minor rearrangements in groupings of cyathostome species in the trees generated using the ITS-2 and l-rRNA sequences. *T. gobi* was found to be grouped within the large clade of cyathostomes in the l-rRNA tree (Fig. 2). In the ITS-2 trees, however, it grouped external to the cyathostomes but still associated with the clade. In order to investigate whether a better phylogenetic resolution could be achieved, it was decided to determine if the data partitions were congruent and could be combined. A conditional data combination approach (Bull *et al*. (1993; DeQueiroz, Donaghue & Kim, 1995; Huelsenbeck, Bull & Cunningham, 1996) was adopted to analyse the mitochondrial and nuclear data sets. This method involves analysis of separate data sets, termed process partitions (Bull *et*

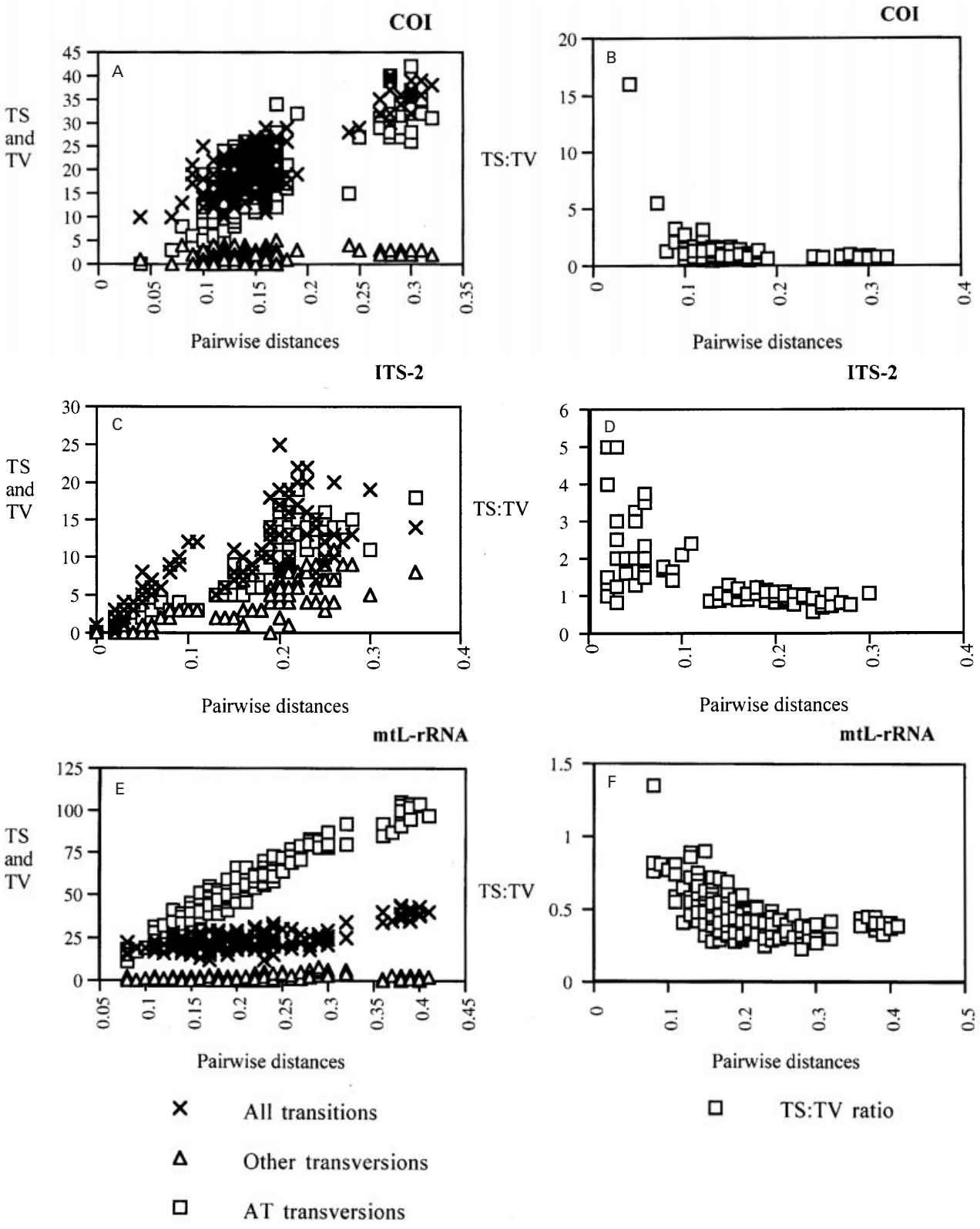

Fig. 1. (A–F).Transitions, AT transversions and all other substitution types for each gene plotted against pairwise distances calculated under ML using a GTR model with starting parameters estimated from the data (A, C, E) in PAUP*4.0d64. Transition to transversion ratios for each gene are plotted against pairwise distances using a GTR model under ML (B, D, F) in PAUP*4.0d64.

al. 1993), followed by a test for heterogeneity among partitions. If there is no significant heterogeneity between partitions, then all the data are combined and analysed. Tests were performed to determine if there was heterogeneity between data sets and to assess the phylogenetic signal from each gene. Tree length distribution analysis (Hillis & Huelsenbeck, 1992) was performed in order to examine the

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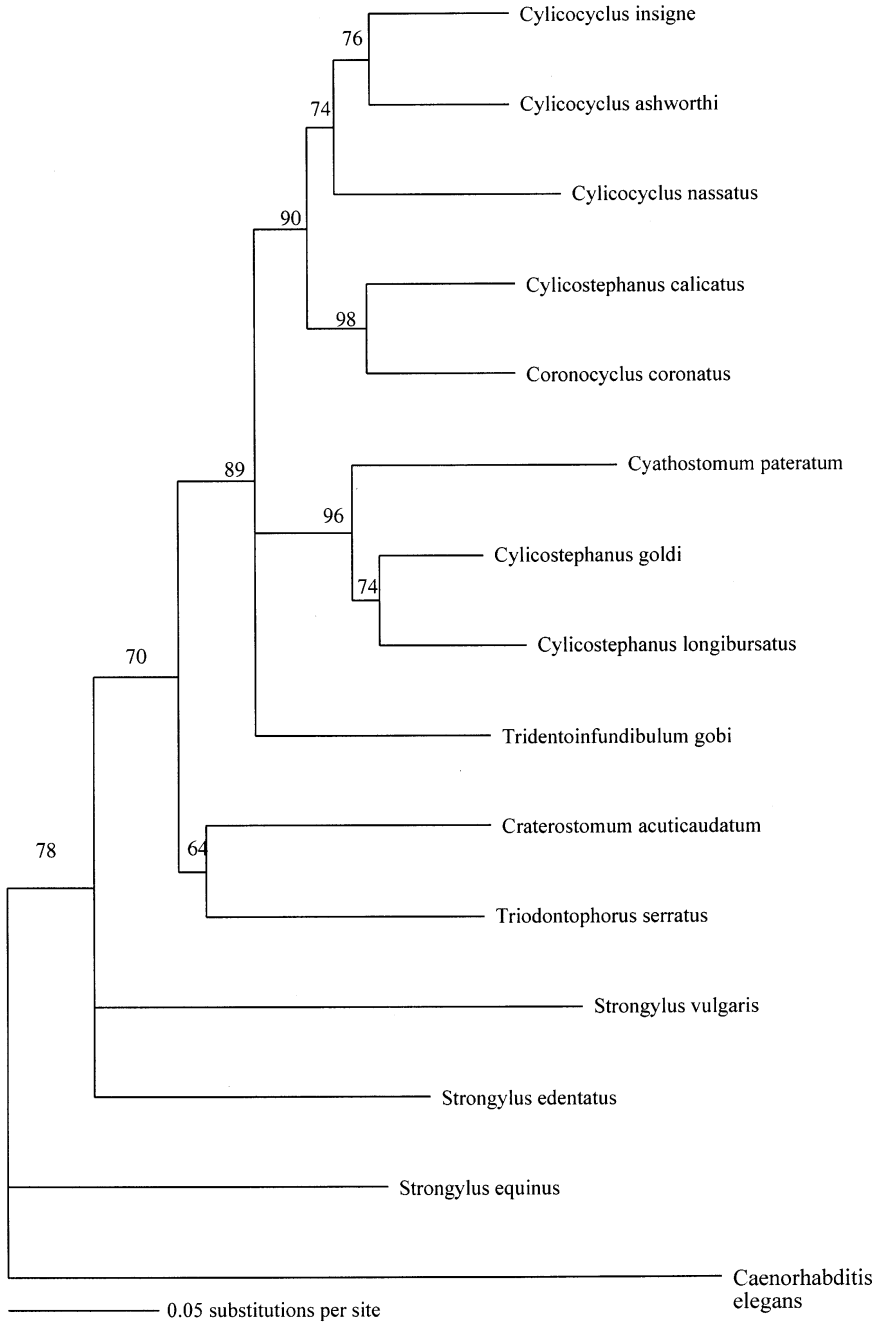

Fig. 2. Phylogram of the l-rRNA data set using Maximum Likelihood, (under a GTR model with starting parameters estimated from the data in PAUP*4.0d64), depicting the evolutionary relationships among the Cyathostominae and Strongylinae. *Caenorhabditis elegans* is the outgroup for the tree. Numbers above branches represent bootstrap values.

phylogenetic content of each data set. The g1 statistic (Sokhal & Rolf, 1981) was used to provide a conventional measure of skewness for tree-length distribution. The tree length distributions and the g1 values for each data set (l-rRNA and ITS-2) were determined in PAUP*4.0d64 (Swofford, 1998). The significance of the g1 statistic was assessed using the critical values calculated by Hillis & Huelsenbeck (1992).

Advocates of conditional combination have argued that testing for incongruence between data partitions is an important step in data exploration. Several statistical tests of incongruence exist (Templeton, 1983; Rodrigo *et al*. 1993; Huelsenbeck *et al*. 1996). The Incongruence Length Difference (ILD) test (Mickevich & Farris, 1981) was employed in the current study. This test (known as the Partition Homogeneity test in PAUP*4.0d64) has been reported to be most useful in distinguishing between some cases in which combining the data generally improved phylogenetic accuracy and other cases in which the accuracy of data combination was poorer compared with the individual partitions (Cunningham, 1997). The difference between the numbers of steps required by individual and combined analysis is the ILD. A partition homogeneity

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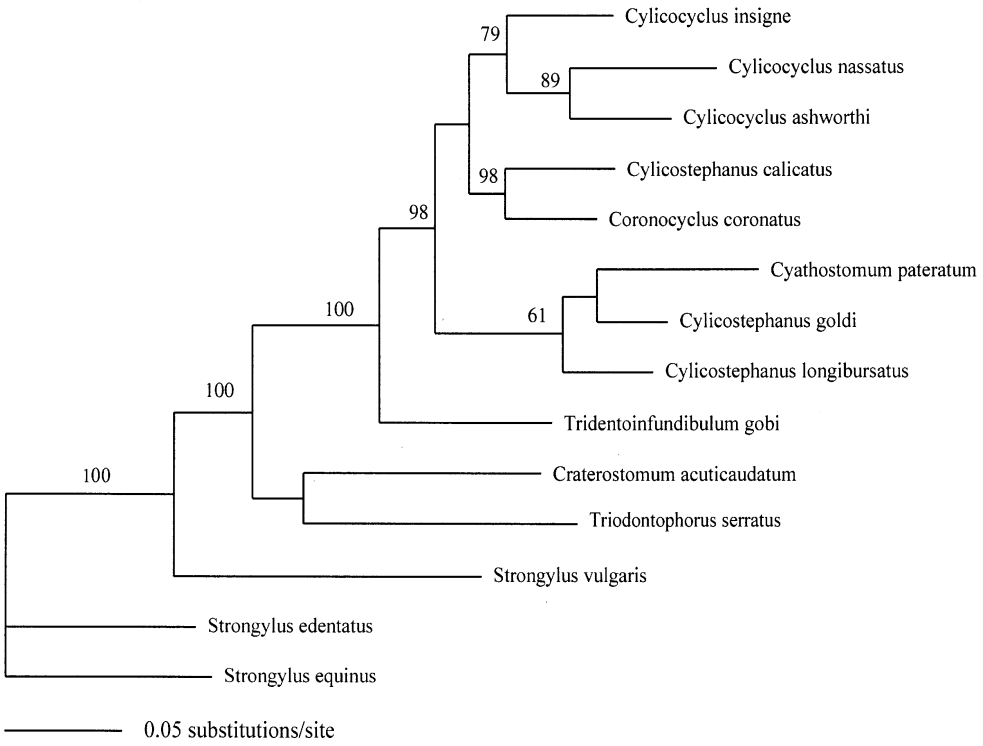

Fig. 3. Phylogram of combined analysis of the mitochondrial l-rRNA and nuclear ITS-2 sequences using Maximum Likelihood, (under a GTR model with starting parameters estimated from the data in PAUP*4.0d64), depicting the evolutionary relationships among the Cyathostominae and Strongylinae. Numbers above branches represent bootstrap values.

test was performed (as implemented in PAUP*4.0d64) to examine differences between each gene and to assess if there was sufficient homogeneity between the two data sets to perform a combined analysis.

The result of each tree length distribution for each data set individually and for the combined data set showed that all values of g1 were estimated to be statistically significant at $P < 0{\cdot}01$ (Hillis & Huelsenbeck, 1992). Comparing g1 values among the two data partitions, the l-rRNA had a g1 $= -0{\cdot}54$ while the ITS-2 showed a stronger left skew at g1 $= -1{\cdot}78$.

The results of the partition homogeneity test showed that the l-rRNA data appeared to be congruent with the ITS-2 ($P = 0{\cdot}23$). Where pairwise comparisons show significant homogeneity, the P value is usually greater than 0·01.

Combined analysis

Phylogenetic analysis of the combined l-rRNA/ITS-2 data set using NJ, MP and ML generated trees with identical topology. For highly composition-biased and rapidly saturating sequences, the ML approach has been suggested to be the most appropriate in nematodes (Blouin *et al*. 1998). Figure 3 shows the ML tree generated using a GTR model estimated from the data.

Pairwise evolutionary distances calculated under ML with a GTR model estimated from the data, ranged from 8 to 17 % among cyathostome species. Inter-specific differences between congeneric species were high; e.g. 9·5 % between *C. ashworthi* and *C. nassatus*, 11·2 % between *C. nassatus* and *C. insigne* and 9 % between *C. insigne* and *C. ashworthi*. This was as high as the inter-specific differences between some members of different genera, e.g. 11·7 % inter-specific difference between *C. insigne* and *C. goldi*. *T. gobi* differed from all cyathostome species by 12·8–14·6 %, however, this was within the same magnitude of difference as that between cyathostome species. *Craterostomum acuticaudatum* differed from the cyathostomes and *Strongylus* spp. by 18·2–22·1 %. Among the large strongyle species, inter-specific differences ranged from 15·3 % (*S. edentatus* versus *S. equinus*) to 23·1–25·4 % (*S. vulgaris* versus *S. edentatus*, and *S. vulgaris* versus *S. equinus* respectively). The cyathostome species differed from *Strongylus* spp. by 21–26·4 %.

DISCUSSION

In the current study, the ITS-2 and l-rRNA genes were shown to be more appropriate than the COI gene for use in phylogeny reconstruction. These non-coding sequences/regions are under fewer selection and functional constraints than protein-coding genes such as COI (Hillis & Dixon, 1991) which was highly conserved. The inability of the COI sequence to provide a good phylogenetic resolution was also compounded by the fact that

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there was strong A+T bias, which poses problems for phylogeny reconstruction in nematodes (Blouin *et al*. 1998). Due to the A+T bias, sites will saturate prematurely as there are fewer possible character states, reducing resolution in the deeper parts of trees, especially when distance or parsimony methods are used (Brower & DeSalle, 1998). Blouin *et al*. (1998) suggested that these two problems could be minimized by restricting use of mtDNA coding gene sequences for phylogenetics only to relatively closely related nematode species.

The high A+T bias in the nematode genome was reflected in the A+T composition of the 3 genes studied. In the COI gene, the A+T composition of the first and second position sites was lower than that of the third position sites; a finding consistent with mutational pressure (Blouin *et al*. 1998). Transitions and A↔T transversions appeared to saturate as more distantly related species were compared. Blouin *et al*. (1998) reported that in intraspecific comparisons, each transition was 5–6 times more common than the most abundant transversion, A↔T. However, when species were compared within a genus, the number of A↔T transversions had outpaced transitions, as reported here. Thomas & Wilson (1991) also reported a rapid decrease in TS:TV ratios from intraspecific to interspecific comparisons in *Caenorhabditis*, consistent with the findings reported in the present study.

It is interesting to note that all members of the Cyathostominae and Strongylidae appeared to be genetically diverse (as evidenced by the values obtained for pairwise distances for the COI gene and for combined analysis of all the genes). Based on analysis of the nuclear s-rRNA gene, Blaxter *et al*. (1998) noted that although the Strongylida (parasitic in vertebrates) were morphologically more disparate than the free-living Rhabdidita, they showed very little genetic divergence compared to the latter. The relatively high values for pairwise evolutionary distances between members of the Strongylida reported here, based on analysis of the COI and l-rRNA sequences, reflects high rate of substitution in these genes compared to nuclear ribosomal genes such as the s-rRNA, making them more useful in resolving relationships at genus or family level rather than at the congeneric level (Liu, Berry & Moldenke, 1997). The strongylids of horses probably represent ancient lineages in hosts that may be relicts of formerly larger groups of species, many of which are now extinct (Durette-Desset *et al*. 1994). This may also account for the greater level of genetic divergence found for equine strongylids.

The combined analysis of l-rRNA/ITS-2 generated trees with groupings more strongly supported statistically by bootstrap analysis than those generated by analysis of each region separately. Phylogeny based on a single molecule may not accurately reflect the history of all the species studied (Blaxter *et al*. 1998) and information from multiple unlinked genetic loci tends to produce more robust estimates of phylogeny and testable hypotheses (De Queiroz *et al*. 1995). Combination of the 2 data sets resulted in a greater number of phylogenetically informative characters and the 3 methods of analysis (NJ, MP, ML) produced trees which were more similar to each other than when the individual data sets were combined. It is important to establish congruence before combining different data sets as it is possible that combining heterogeneously evolving data sets may worsen phylogenetic estimates, making it more difficult to know which of the reconstructed trees would appear to be the most appropriate when the true tree is unknown (Funk *et al*. 1995). Nonetheless, topological agreement among data sets evolving under disparate rules (i.e. the nuclear ITS-2 and the mitochondrial l-rRNA sequences) is a means of phylogenetic corroboration. The use of the GTR model with starting parameters estimated from the data was considered to be the most appropriate method for analysis of the highly A+T biased sequences of these nematodes as it took into account the biased nucleotide composition and the rate difference between TS and TV (Blouin *et al*. 1998).

Strongylus equinus was chosen as the taxon upon which the tree was rooted for the combined analysis of the l-rRNA and ITS-2 genes, based on the results obtained by previous phylogenetic analysis of a larger data set for the l-rRNA. In that separate analysis of the l-rRNA sequences, the free-living nematode, *C. elegans*, was used as an outgroup as it is considered to be relatively closely related to vertebrate parasites of the order Strongylida (Blaxter *et al*. 1998). *S. equinus* was consistently the next most basal taxon to *C. elegans* in that analysis using NJ, MP and ML methods and appeared to be a suitable choice to root the trees for combined analysis of the l-rRNA/ITS-2 data set. In a separate analysis of the ITS-2 gene, it had not been possible, due to a lack of sequence similarity, to accurately align the ITS-2 sequence of *C. elegans* (Ellis, Sulston & Coulson, 1986) with those of the other nematode taxa sequenced here. Therefore it could not be included in the combined analysis.

The separate l-rRNA and ITS-2 data sets and the combined l-rRNA/ITS-2 data set supported a monophyletic clade of the cyathostome species with *T. gobi*. This is the first time *T. gobi* has been reported in Scotland. This species has been reported previously in Asia, North America and Europe (Lichtenfels *et al*. 1998). It has only recently been described in sufficient detail to be recognized (Dvojnos & Kharchenko, 1994). It was described as *Cylicostephanus torbertae* (Lichtenfels & Klei, 1988) in North America. Based in part on preliminary results from the present study, *T. gobi* was classified at the Sun City International Workshop on the

Systematics of Cyathostominea (Lichtenfels *et al*. 1998) in an independent genus, but with uncertain relationships, rather than within *Cylicostephanus*. The separate l-rRNA and the ITS-2 sequences and the combined l-rRNA and ITS-2 sequences of this study support a monophyletic clade including *T. gobi* with the other Cyathostominea but separate from the 3 species representing the genus *Cylicostephanus*. These results are in agreement with the phylogenetic studies of Hung *et al*. (2000) based on a combined constrained analysis of ITS-1 and ITS-2 data sets. Those authors found *T. gobi* to be an independent taxon grouped in a clade with either *Cylicodontophorus bicoronatus* or (50 % majority rule) with *Cylicostephanus bidentatus*, 2 species not included in our analysis.

Molecular analysis failed to provide strong evidence for the separation of cyathostome species into their classical genera, i.e. *Cyathostomum*, *Cylicocyclus*, *Coronocyclus* and *Cylicostephanus*. The current analysis grouped the 3 species of the genus *Cylicocyclus* included within our study in a single clade, separated from 5 species representing the genera *Cyathostomum*, *Cylicostephanus* and *Coronocyclus*. However, our analysis did not resolve the 5 species representing the latter 3 genera. The 3 species of *Cylicocyclus* included in our analysis also grouped together, in accordance with the studies of Hung *et al*. (2000) but that study also failed, despite testing more species (total of 10 species for the 3 genera), to provide support for the genera *Cyathostomum*, *Cylicostephanus* or *Coronocyclus*. In our analysis, *Coronocyclus coronatus* formed a clade with *Cylicocstephanus calicatus*. *Cyathostomum pateratum* formed a clade with 2 species morphologically similar to each other, *Cylicostephanus longibursatus* and *Cylicostephanus goldi*, in agreement with the phylogeny of Hung *et al*. (2000), but that study also included *Cyathostomum catinatum* in a subclade with the very similar species, *C. pateratum*. Our study included only 3 species of *Cylicostephanus*. The closely similar species *Cylicostephanus longibursatus* and *Cylicostephanus goldi* were in the same clade but *C. longibursatus* was more similar to *Cyathostomum pateratum*. *Cylicostephanus calicatus*, in our analysis, grouped with *Coronocyclus coronatus* in a separate clade from the 2 other species of *Cylicostephanus*. Other species of the genus *Cylicostephanus* (*C. bidentatus* and *C. minutus*) studied by Hung *et al*. (2000) did not group together or with the 3 other species of *Cylicostephanus* included in that study. We believe our results and those of Hung *et al*. (2000) indicate the genus *Cylicostephanus* needs further revision. However, a phylogenetic reconstruction, based on morphology is required before this can be undertaken.

The present study provides some evidence of relationships among representatives of the Cyathostominae and the Strongylinae of horses. According to the classification by Lichtenfels (1975, 1980), the small strongyles (or nematodes with small cylindrical buccal capsules) of horses belong to the tribe Cyathostominea of the subfamily Cyathostominae, and those with large globular or subglobular buccal capsules belong to the family Strongylinae. Lichtenfels (1986) emphasized that this separation of the nematodes of the family Strongylinae was arbitrary and needed to be tested. Representatives of the Strongylinae (*sensu* Lichtenfels, 1980) in our study included 3 species of the genus *Strongylus* and *Triodontophorus serratus* and *Craterostomum acuticaudatum*. The 3 genera of large strongyle grouped outside the large clade containing the cyathostomes and *T. gobi*. *T. serratus* and *C. acuticaudatum* were found to be more similar to the Cyathostominea than to species of *Strongylus*, and they grouped together but separately from the representatives of the Cyathostominea. Hung *et al*. (2000) found a similar relationship for these genera in a phylogenetic analysis of the combined sequences of ITS-1 and ITS-2. *Strongylus equinus* and *S. edentatus* appeared to be more closely related (based on pairwise evolutionary distances and phylogenetic analysis) to each other than to *S. vulgaris*. A similar relationship for the 3 species of *Strongylus* was also reported by Hung *et al*. (2000). Our findings also agree with those of Campbell *et al*. (1995) based on analysis of the ITS-2 sequence. The results of the current study illustrate the need for revision of the morphological taxonomy.

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