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RELATIONSHIPS OF NEMATOMORUS SPECIES AND NEMATOMORUS BATTUS ISOLATES (NEMATODA: TRICHOSTRONGYLOIDEA) BASED ON NUCLEAR RIBOSOMAL DNA SEQUENCES

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ABSTRACT: Nuclear ribosomal sequence data from the internal transcribed spacers (ITS-1 and ITS-2), 5.8S subunit, and regions of the 18S and 28S genes were used to investigate sequence diversity among geographic samples of Nematodirus battus, and to infer phylogenetic relationships among Nematodirus species. Phylogenetic analysis of these data yielded strong support for relationships among species, depicting Nematodirus helvetianus and Nematodirus spathiger as sister-taxa and a clade of these 2 species and Nematodirus filicollis. This tree is consistent with caprine bovids as ancestral hosts, with a subsequent host shift to Bovinidae in N. helvetianus. Eleven of 14 N. battus sequences were unique, with 19 variable sites among sequences representing 5 geographic samples. The lowest number of variable nucleotide sites was observed in samples representing apparently recent introductions to the United States and Canada, which is consistent with a population bottleneck concomitant with translocation. Comparison of directly sequenced polymerase chain reaction products and clones revealed evidence for intraspecific variation at some of the sequence sites, and this pattern of variation and that within geographic samples indicates incomplete rDNA repeat homogenization within species. This pattern of variation is not conducive for inferring phylogenetic relationships among sequences representing N. battus or addressing the putative history of introduction.

Nematodirus Ransom, 1907 contains more than 45 described species (Kulmamatov, 1974), and several are characteristic and widespread parasites of domesticated ruminants (Lichtenfels and Pillett, 1983). Nematodirus battus Crofton and Thomas, 1951, a pathogen of sheep, although originally described based on specimens collected from Scotland (Crofton and Thomas, 1951, 1954), has subsequently been reported from numerous localities in western Europe and more recently in the Western Hemisphere (reviewed in Hoberg, 1997; Rickard et al., 1997). The current geographic distribution of N. battus is assumed to be linked to dissemination and sequential establishment following translocation and introduction, e.g., from Great Britain to Norway, among countries in western Europe, and from Great Britain to Canada and the United States, with chronically infected adult sheep (Hoberg, 1997). The apparently abrupt appearance and recognition of the parasite in Great Britain, however, has never been adequately explained (Crofton and Thomas, 1951, 1954; Jansen, 1973; Hoberg et al., 1985). In addition, enigmatic reports of isolated and endemic foci of N. battus in Italy and possibly Yugoslavia (Lepojev, 1963; Nardi et al., 1974) defy simple explanation. The phylogenetic relationship of N. battus to other species of Nematodirus has also remained unresolved, although Jansen (1973) suggested affinities with species that parasitize Cervidae, a contention corroborated in part by a taxonomically limited phylogenetic analysis of the genus (Hoberg, 1997). Assessments of overall genetic similarity based on rDNA sequences have showed greatest pairwise similarity between Nematodirus spathiger and Nematodirus helvetianus, and represented N. battus as most genetically distinct in comparison to 3 congeners (Newton et al., 1998).

There have been few attempts to test critically the hypothesis of a recent history of introduction for N. battus in western Europe and North America. Specimens of N. battus representing apparently discrete populations from Great Britain, Norway, Canada, and the United States are morphologically indistinguishable (Hoberg et al., 1986). Comparative analysis of soluble proteins by isoelectric focusing (IEF) for populations of N. battus representing Oregon, eastern Canada, and the United Kingdom revealed distinct protein profiles for isolates, but a high level of overall similarity among these isolates. Due to the difficulty of inferring homology among IEF bands, however, these data were not useful for assessing phylogenetic relationships or the potential history of introduction (Rickard et al., 1997).

In the present study, we sequenced nuclear ribosomal DNA (rDNA; internal transcribed spacer-1 [ITS-1], 5.8S subunit, ITS-2, and parts of the flanking 18S and 28S genes), and used trees inferred from parsimony and distance analyses to: (1) evaluate the phylogenetic relationship of N. battus to other commonly occurring species found in domesticated ruminants, including Nematodirus filicollis Rudolphi, 1802, N. spathiger Railliet, 1896, and N. helvetianus May, 1920; (2) assess the sequence diversity and relationships among 5 population samples of N. battus from the western Palearctic and North America as an initial test of the hypothesis for sequential geographic introduction and emergence; (3) investigate the geographic source population for N. battus in the United States; and (4) evaluate the diagnostic potential of rDNA sequences for these Nematodirus taxa.

MATERIALS AND METHODS

Source and history of specimens

Voucher specimens representing each of the Nematodirus species and populations examined were deposited in the U.S. National Parasite Collection (USNPC), Biosystematics and National Parasite Collection Unit (BNPCU), U.S. Department of Agriculture (USDA), Agricultural Research Service. A limited number of frozen (−70 C) specimens have been maintained in ultralow storage at the USNPC. Specimens from foreign sources were imported under specific permits issued by the Animal and Plant Health Inspection Service, USDA.

Specimens were collected from naturally infected sheep Ovis aries L. from the Willamette Valley, adjacent to Corvallis, Oregon during...
fitted primers (primer numbers followed by sequence positions according to Caenorhabditis elegans numbering; Ellis et al. [1986]; GenBank X03680) for PCR were designed based on alignments of the 18S end primer (primer no. 93, 2633–2662 5′-TTAGGCCGGATCCCAATTG-3′ and primer no. 94, 3745–3764 5′-TTAGTTTCTTTTCTTTCGCTG) for the following nematodes: Strongyloides stercoralis (18S, Putland et al. [1993]; M84229), N. battus (18S, Zarlenga et al. [1994]; U01230), C. elegans (28S, Ellis et al. [1986]; X03680), Brugia pahangi (28S, Qu et al. [1986]; M15409), Nematospiroides dubius (28S, Qu et al. [1986]; M15310), and Onchocerca gibsoni (28S, Qu et al. [1986]; M15308). The 18S primer was designed to mismatch vertebrate (human) sequence (McCallum and Maden, 1985; X03205) so that potential host and laboratory nucleic acid contamination would be less likely to amplify by PCR. For PCR amplifications that were subsequently used for cloning, Taq polymerase was employed with a final MgCl2 concentration of 3 mM. Cycling conditions included an initial DNA denaturation at 94°C for 4 min followed by 25 cycles of 94°C for 30 sec, 48°C for 30 sec, and 72°C for 75 sec, and a postamplification extension at 72°C for 5 min. A 2-μl aliquot of the 50-μl amplification product was subjected to agarose gel electrophoresis to confirm product size (approximately 900 bp) and yield. Prior to cloning, the remainder of the sample was washed 3× by spin filtration (Millipore Ultrafree-MC 30,000 NMWL; Bedford, Massachusetts) using 350 μl TE, pH 8.0 (modified to 0.1 M EDTA), and reconstituted to 48 μl by vacuum evaporation. Seven and one-half micro liters of washed PCR product was used for ligation into pGEM-T vector (Promega, Madison, Wisconsin) and used to transform DH5α Escherichia coli. DNA sequencing templates for clones were prepared by CsCl purification of plasmid DNA from 100 μl Luria–Bertani broth cultures. Two clones were sequenced for each species and geographic isolate of Nematodirus. Clones were sequenced manually using the Del-ta-Taq cycle sequencing kit (Amersham, Piscataway, New Jersey) and α-32P-ATP (specific activity >3,000 Ci/mmol), with annealing temperatures for the labeling and termination steps adjusted empirically for each sequencing primer. 7-Deaza-dGTP was used in cycle and termination sequencing mixes to reduce gel compression artifacts resulting from template secondary structure. Sequences were fully double-stranded for each clone and obtained using pGEM-T vector sequencing primers of our own design (primer no. 156, pGEM-T-2974–2997 5′-GCGGACTTGAATTTATCAGGAT-3′, primer no. 157, pGEM-T-110–135 5′-GAC-TATAGAAATATCTCAAGCTACG-3′), and 4 internal rDNA primers (no. 656, 5′-GCTGTCGGAGTTCTTAAAGTACGCGG-3′, no. 389, 5′-TCGAGGGCTTATAGGTGCCTG-3′, no. 264, 5′-CCTTTCCTATCCTCAGGATGG-3′, and 5′-CCTTTCCTATCCTCAGGATGG-3′) sequence data obtained using vector primers. Three of the 4 internal primers annealed to regions that were not invariant among the Nema-todirus taxa. To test for intravidual rDNA variation, PCR product amplified from DNA of individual males was sequenced directly. PCR amplifications from individual males were performed using a proof-reading DNA polymerase (ID Proof, ID Labs Biotechnology); amplification and cycling conditions were as previously described except a 38°C annealing temperature was used. Proofreading polymerase and a 58°C annealing temperature were also used to obtain PCR products for digestion with restriction endonucleases. Sequence of the U. stenocephala outgroup was obtained by direct sequencing of PCR product as described for Nematodirus individuals using the amplification primers (93 and 94) and internal primers 264 and 389. Templates for direct PCR sequencing were prepared by enzymatic treatment using exonuclease I and shrimp alkaline phosphatase (PCR product Presequencing Kit, Amersham). Sequences were obtained from PCR templates using BigDye (Perkin-Elmer, Norwalk, Connecticut) terminator cycle se- quencing chemistry and an ABI 377 DNA Sequencer. For the clones, PCR template sequences were completely double-stranded using the sequencing primers described previously. Automated sequencing was substituted for manual methods due to its availability and efficiency rather than concerns regarding accuracy.

**Sequence analysis**

Invariable flanking regions corresponding to the PCR amplification primers were removed from the sequences prior to multiple sequence alignment and phylogenetic analysis because primer incorporation dur-
ing amplification masks potential mismatches (substitutions) that may occur in priming regions. Nematodirus sequences were aligned initially using CLUSTAL X (Thompson et al., 1997), and the resulting output was adjusted manually to increase sequence similarity; this yielded an alignment of 869 characters (=standard alignment; ITS-1 and ITS-2 regions shown in Fig. 1). CLUSTAL X was used to produce 2 profile alignments, keeping the standard alignment for Nematodirus taxa constant, and sequentially adding the outgroups U. stenocephala (Ancylostoma tomatoidae) and C. elegans (Rhabditoidae). The alignment was adjusted by removing a unique 102-nucleotide (nt) insertion in the 3′ end of the C. elegans 28S sequence, and manually adjusting the alignment for the 2 outgroups at 3 sites (involving a total of 4 nt), yielding a dataset of 1,000 characters. For maximum parsimony (MP) analyses, unambiguous contiguous gaps were recoded such that “gap” represented 1 character and “nucleotide present” was coded as the alternative character state (Swofford, 1993; Crandall and Fitzpatrick, 1996). This conservative gap-recoding procedure yielded 32 additional characters in the standard alignment with outgroups. Eighteen of these characters involved an indel separating C. elegans from other taxa; 6 recoded gaps were parsimony informative, and 1 recoded site included parsimony-informative variation within the ingroup taxa (Fig. 1, site 376). The effect of alignment ambiguity on the analyses was explored by producing 2 additional data matrices (truncated alignments, 1 with gap recoding) that excluded regions where inferences of positional homology were judged to be tenuous (15 regions, 89 characters in the standard alignment with outgroups). In the truncated gap-recoded alignment, regions containing 6 of the recoded indels were removed.

Data were analyzed by 2 tree inference methods: MP and neighbor joining (NJ) using PAUP* 4.0 (beta version 4.0b2; Swofford, 1998). For all analyses, identical sequences in the alignments were treated as single terminal taxa. Parsimony trees were obtained using a heuristic search (simple stepwise addition, TBR branch-swapping, MULPARS, steepest descent, simple stepwise addition, and maxtrees set to 60,000). Gap-recoded datasets were used for MP analyses, whereas the unrecoded datasets were used for NJ analyses. Unrecoded gaps were treated as missing data in MP analyses. Reported consistency and homoplasy indices (CI, HI) do not include uninformative characters. Bootstrap MP trees (1,000 replicates) were also produced using heuristic searches (TBR branch-swapping, MULPARS, steepest descent, simple stepwise addition, and maxtrees of 100 per replicate). Log-determinant distances were used for NJ analyses. Templeton’s modified parsimony test (Templeton, 1983), as executed in PAUP* 4.0, was used to compare the sequence-based phylogenetic hypothesis with an alternative based on analysis of morphological characters (Hoberg, 1997).

RESULTS

Intra- and interspecific sequence differentiation

Nucleotide sequences for clones and PCR products of Nematodirus taxa and U. stenocephala were deposited in GenBank (accession numbers AF194123–194145). Sequences from clones (Fig. 1) revealed differences among and within geographic isolates of N. battus and within single isolates of other species. For N. battus, 19 sites showed variation, not counting 3 sites with indels. Differences between the 2 clones represent each geographic sample of N. battus generally showed more transition (ti) substitutions and indels (in) than transversions (tv): Norway, 5 ti, 3 in, 2 tv; Oregon, 1 ti; PEI, 1 tv; St. Kilda, 7 ti, 2 in; Weybridge, 6 ti. For the other 3 species, transitions were observed exclusively between clones: N. filicollis, 1 ti; N. helvetianus, 1 ti; N. spathiger, 2 ti. The presence of length variation affected the ability to successfully sequence PCR products directly. Direct automated sequencing of PCR amplicons from individual male specimens was successful for 6 of the 8 taxa. Nematodirus filicollis and N. battus St. Kilda failed to yield readable sequence for specific regions of their PCR amplicons. These failures were reproducible and the point of failure consistent (within each taxon) among amplicons obtained from different individual males. Direct amplicon sequencing of the St. Kilda individuals failed to yield readable sequence beginning at the site corresponding to the position of the first insertion–deletion observed between the 2 clones (Fig. 1, position 443 or 463, depending on orientation of the sequencing primer used). Nucleotides in the double bands of the resulting electropherograms corresponded to that predicted from the clone sequences and the expected readshift due to length differences. The PCR sequencing failure in the N. filicollis individuals also appeared consistent with 1 or more indels and a mixed population of PCR products, yet the 2 clones sampled for this species were of the same length.

Sequence polymorphisms were observed for directly sequenced PCR products (amplified using proofreading polymerase) at 15 sites among 4 taxa (Fig. 1). For all of these sites, 2 nucleotides with approximately equal electropherogram peak heights (“heterozygotes”) were observed in both sequenced strands. For 5 of these sites, the polymorphisms represented the 2 alternative nucleotides observed at the respective sites in clones of the same species or geographic isolate (3 sites with ti, 2 sites with tv). For the remaining 10 polymorphisms where no differences were detected between the 2 sampled clones, 6 involved ti and 4 tv. For each of these 10 polymorphisms, the nucleotide observed in the clones corresponded to 1 of the 2 detected in the PCR amplicon. None of these polymorphic sites was shared between species; sites 67 and 307 were polymorphic in isolates of N. battus from 2 geographic localities (Norway/Weybridge and Oregon/Weybridge, respectively). Among N. battus, Norway clones showed the most variation (5 ti, 2 tv, and 3 indels); the largest of the indels was a 4-tt direct repeat (Fig. 1, sites 311–314) shared with the St. Kilda clones. The Norway direct PCR amplicon sequence did not show sequence ambiguities or base-calling difficulties at these sites, although the PCR sequence differed from both clones at 1 site (site 447, a transition).

Pairwise comparisons revealed that 3 N. battus sequences were identical in the standard alignment (PEI PCR, PEI cl 1, and Oregon cl 2). These 3 sequences were treated as 1 terminal taxon in all analyses. Differences among all sequences representing each geographic isolate or species (but not considering sites polymorphic in PCR amplicon sequences as different if they match a base in a clone) were as follows: N. battus Norway, all 3 sequences different (14 variable sites); N. battus Oregon, PCR same as Oregon cl 1 (1 variable site); N. battus PEI PCR same as PEI cl 1 (1 variable site); N. battus St. Kilda, both clones different (9 variable sites); N. battus Weybridge, PCR same as Weybridge cl 4 (6 variable sites); N. spathiger, all sequences different (2 variable sites); N. helvetianus, PCR same as cl 1 (1 variable site); N. filicollis, both clones different (1 variable site).

 Parsimony and NJ analyses

Parsimony analysis of the standard gap-recoded alignment did not reach completion due to the large number of equally parsimonious trees recovered (exceeding the maxtrees setting of 60,000). The strict consensus of the 60,000 saved trees (671 steps, Cl 0.792, HI 0.208) revealed that Nematodirus species were diagnosed by apomorphies and were distinct (Fig. 2); bootstrap support for species of Nematodirus was 99% or greater in all cases.
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**Figure 1.** Alignment of ITS-1 (1–390) and ITS-2 (391–627) sequences (standard alignment) of *Nematodirus* spp. Not shown are 40 nt of 3'-end 18S sequence, 152 nt of 5.8S sequence, and 50 nt of 5'-end 28S sequence. †Sequences identical for *N. battus* Oregon cl 2, *N. battus* PEI PCR, *N. battus* PEI cl 1, and *N. battus* PEI cl 3, with only Oregon cl 2 shown. The 18S and 28S sequences were invariant; the 5.8S sequence had 3 sites with substitutions, and 1 of these was parsimony informative. The complete dataset is deposited in TreeBase. Highlighted sites in directly sequenced PCR amplicons had 2 nt as evidenced by both sequence strands (K/T, G/A, C/T).

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**Figure 1.** Alignment of ITS-1 (1–390) and ITS-2 (391–627) sequences (standard alignment) of *Nematodirus* spp. Not shown are 40 nt of 3'-end 18S sequence, 152 nt of 5.8S sequence, and 50 nt of 5'-end 28S sequence. †Sequences identical for *N. battus* Oregon cl 2, *N. battus* PEI PCR, *N. battus* PEI cl 1, and *N. battus* PEI cl 3, with only Oregon cl 2 shown. The 18S and 28S sequences were invariant; the 5.8S sequence had 3 sites with substitutions, and 1 of these was parsimony informative. The complete dataset is deposited in TreeBase. Highlighted sites in directly sequenced PCR amplicons had 2 nt as evidenced by both sequence strands (K/T, G/A, C/T). Complete alignment with *U. stenocephala* and *C. elegans*, including 18S, 28S, and 5.8S sequences is deposited in TreeBase.
The topology of the bootstrap parsimony tree was almost identical to Figure 2, with the addition of a poorly supported (54%) clade of *helvetianus* cl 1 and *helvetianus* PCR. There was 100% bootstrap MP support for a clade consisting of *N. helvetianus*, *N. spathiger*, and *N. flicollis*, and within this group, 100% support for a sister-taxon relationship between *N. helvetianus* and *N. spathiger*. An alternative topology (((*N. battus, N. helvetianus*), *N. spathiger*), *N. flicollis* root among ingroup) consistent with the morphologically based phylogenetic hypothesis of Hoberg (1997) was significantly worse (*P* < 0.0001) than the most parsimonious topologies (alternative topology 703 steps, CI 0.725, HI 0.275) as assessed by Templeton's parsimony test.
There was little cladistic structure among sequences of *N. battus* and no evidence for monophyletic groups structured by geographic locality. Cladistic structure within *N. battus* sequences was limited to a sister-group relationship between Norway cl 3 and St. Kilda cl 8, and a clade consisting of all *battus* sequences exclusive of Weybridge cl 5. These relationships were recovered in the strict consensus and bootstrap consensus trees (Fig. 2); however, only the Norway cl 3 and St. Kilda cl 8 clade received moderate to high bootstrap support. Parsimony analysis of the truncated gap-recoded alignment reached completion and yielded 612 equally parsimonious trees (596 steps, CI 0.792, HI 0.208). The strict consensus of these trees yielded

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the same topology with respect to species relationships as Figure 2; however, sequences representing geographic populations of *N. battus* were collapsed in a polytomy.

The NJ analysis of log-determinant distances yielded a dichotomous phylogram with very short internal branches within species and moderate branch lengths among *Nematodirus* species (Fig. 3). Branch lengths from the root of the tree to *Nematodirus* species did not show marked differences (Fig. 3). All nodes that were strongly supported in the bootstrap MP analyses were also recovered and strongly supported in the NJ bootstrap tree. Among intraspecific sequence comparisons, only the clades Norway cl 3 plus St. Kilda cl 8, *helvetianus* PCR plus *helvetianus* cl 4, and *spathiger* cl 4 plus *spathiger* cl 5 received moderate to high bootstrap support. For relationships among *N. battus*, topological differences between the dichotomous NJ trees inferred from the standard and truncated alignment were substantial, and the strict consensus of these 2 trees (Fig. 4) shows that only 2 *N. battus* clades were common to both topologies. In 1 of these clades, all PEI sequences and 1 Oregon sequence (cl 2) are most closely related; in the other clade, the remaining 2 Oregon sequences are grouped with 2 of the 3 Weybridge sequences, although sequences from Oregon and Weybridge are not themselves monophyletic.

**PCR/restriction fragment length polymorphism (RFLP) diagnostics**

Sequences of *Nematodirus* have species-specific diagnostic restriction enzyme recognition sites that yield fragment sizes amenable to detection on standard agarose gels (Fig. 5). The use of 2 restriction enzymes, *Apo* I and *BstE* II, is required to yield diagnostic rDNA fragment profiles for species. Digestion with *Apo* I yields fragment patterns that distinguish *N. spathiger* and *N. helvetianus* (538/361 and 537/359 base pairs [bp], respectively) from *N. filicollis* (405, 365, 132 bp) and *N. battus* (536–542, 237–239, 122–123 bp). *Nematodirus spathiger* and *N. helvetianus* may be distinguished using *BstE* II that does not cut *N. helvetianus* but yields 2 fragments for *N. spathiger* (536 and 363 bp).

**DISCUSSION**

**ITS sequence variation**

Repeat units of multigene families, such as rDNA, typically show more sequence similarity within and among individuals of a species than would be predicted if they were evolving independently, a phenomenon attributed to concerted evolution.
Recent studies, however, have revealed rDNA sequence heterogeneity within nematode species and individuals (e.g., Blok et al., 1997; Powers et al., 1997; Gasser et al., 1998; Heise et al., 1999; Hugall et al., 1999), as has been reported for other organisms (Schlötterer and Tautz, 1994; Polanco et al., 1998). Schlötterer and Tautz (1994) showed that for *Drosophila melanogaster*, individuals within local interbreeding populations are homogeneous for different ITS variants and proposed that the rate of intrachromosomal recombination greatly exceeds that between homologous chromosomes, driving concerted evolution. Gasser et al. (1998) also invoked intrachromosomal exchange to explain patterns of inter- and in-
Figure 3. Neighbor-joining phylogram based on log-determinant distances. Bootstrap values for NJ inference (1,000 replicates) are shown when >60%. Branch lengths are scaled to expected amount of change per site.
Figure 4. Strict consensus of the 2 NJ trees inferred from the standard alignment and the truncated alignment (excluding alignment ambiguous sites), both using log-determinant distances.
traindividual ITS-2 variation among geographic samples of *Haemonchus contortus*, again suggesting that intrachromosomal events such as unequal crossing-over or gene conversion between sister chromatids are most likely responsible for repeat homogenization. For these *Nematodirus* species, PCR polymorphisms within ITS sequences are consistent with the amplification of more than 1 repeat type within some individuals. Documenting the extent of intraindividual variation requires sequencing multiple clones from individual worms and using PCR conditions that enhance the probability that such sequences represent the spectrum of diversity present within an individual. A relatively large number of nucleotide sites (19) were variable within *N. battus*, the only species for which a moderate number of sequences (14) were systematically sampled, although it is also notable that 3 *N. spathiger* sequences had 12 variable sites. This sequence variation far exceeds the potential misincorporation error rate of *Taq* polymerase during PCR (Kwiatowski et al., 1991), and *Taq* error would not explain the co-occurrence of variation at the same sites in comparisons of direct PCR sequence (amplifications from individual nematodes using proofreading polymerase) to clones derived from separate PCR amplifications (using *Taq* polymerase) representing pooled individuals. In addition, each of these sequences was fully double-stranded for determination, making independent and coincident errors in base calling highly unlikely. Observations concerning variation among *Nematodirus* ITS sequences should be considered minimal estimates, because use of different PCR conditions, e.g., presence of denaturants, may allow amplification of sequence variants that are not revealed with standard conditions (Buckler et al., 1997). The observed number of variable sites within *N. battus* and *N. spathiger* far exceeds that reported previously for *N. helvetianus* and *N. filicollis* (Heise et al., 1999) or observed for those same species in our study.

For *N. battus*, 10/19 variable sites were unique to a single sequence, 9 were parsimony informative within this species, and 11/14 sequences (8/10 clones) were unique. The large number of unique sequence variants contributes to the inability to resolve relationships among sequences of *N. battus*. This distribution of sequence variation is consistent with incomplete repeat homogenization within most geographic samples and for 2 of 4 individuals for which PCR amplicons were successfully sequenced. The nature of this variation impacts on the potential use of ITS sequences to represent relationships among geographic samples of *Nematodirus* because, with incomplete repeat homogenization, no single sequence (and particularly no clone) from an individual represents the variation present among copies in the sample. For example, with reference to Figure 2, a misleading conclusion regarding relationships among geographic samples would be made if only Norway cl 3, St. Kilda cl 8, Oregon cl 1, and Weybridge cl 5 were sampled and included in the analysis. Although concerted evolution can homogenize rDNA copies within geographic populations yielding lineage-specific sequences (Hillis and Dixon, 1991; Hillis, 1994), our findings reinforce the importance of investigating repeat heterogeneity within samples if sequences are used to represent terminal taxa (such as geographic samples) in a phylogenetic analysis (Rich et al., 1997).

Newton et al. (1998) reported some additional ITS-2 differences within *Nematodirus* species, including polymorphic sites in direct PCR amplicon sequences. In comparison to our sequences, the *N. filicollis* sequence reported by Newton et al. (GenBank Y14011) had 1 nt difference (Fig. 1, position 401, A for T), and 2 polymorphic sites (positions 477 and 575), wherein 1 of the 2 bases was observed in our 2 clones. A sequence for *N. filicollis* reported by Heise et al. (1999) was the same as determined by us, except they observed the polymorphism reported by Newton et al. (1998) at position 575. The *N. helvetianus* sequence of Newton et al. (Y14013) had 1 difference (position 482, A for G) and 2 polymorphic sites (sites 393, 454), wherein 1 of the 2 bases was observed in our clones and PCR product. The *N. spathiger* sequence of Newton et al. (Y14012) shared the polymorphic site observed in our PCR product sequence (site 436). Comparison of the ITS-2 region for our sequences with those reported by Newton et al. (1998) also revealed 2 likely sequencing errors. Their *N. helvetianus* and *N. spathiger* sequences both had an “S” or C/G at position 441 (instead of G) and omitted the C at position 442. The most likely explanation for this difference is that a sequencing artifact, e.g., a compression, led to scoring “S” instead of G followed by C. This GC sequence corresponding to positions 441 and 442 was also reported by Heise et al. (1999) for *N. helvetianus* but not noted as different from the Newton et al. (1998) sequence.

Newton et al. (1998) also provided restriction maps for the ITS-2 of these 4 *Nematodirus* species and suggested that combinations of endonucleases could be used to distinguish among them. However, many of the potential fragments from these conditions.
ITS-2 digests are too small to be easily resolved with standard agarose electrophoresis, given that the entire ITS-2 region is approximately 230 bp. A more tractable approach is to amplify the entire ITS-1/5.8S/ITS-2 region, and use 2 restriction endonucleases, Apo I and BstE II, to distinguish DNA from these 4 species. This method may prove useful for using DNA isolated from eggs to distinguish among 3 species infecting ruminants (*N. filicollis, N. spathiger, and N. helvetianus*) where identification based on morphology and morphometry of eggs is problematic.

**Relationships among *Nematodirus* spp.**

Phylogenetic analysis strongly supports the hypothesis that samples of sequences from each species of *Nematodirus* are monophyletic, and this indicates that concerted evolution is homogenizing repeats at a rate exceeding speciation. This is prerequisite for recovering the correct species-level relationships, because if concerted evolution operates more slowly than speciation, phylogenetic inference may be confounded by paralogy (Sanderson and Doyle, 1992). Sequence-based inferences of recombination, phylogenetic inference may be confounded by paralogy (Newton et al., 1998) based on ITS-2 sequences showed data are available for only 4. A prior analysis of genetic similarity (Newton et al., 1998) based on ITS-2 sequences showed a low dissimilarity (3.9%) between *N. spathiger* and *N. helvetianus*, a phenetic cluster involving these 2 species and *N. filicollis*, and representation of *N. battus* as most dissimilar by midpoint rooting, or the assumption of rate uniformity of ITS-2 evolution (Newton et al., 1998). The outgroup-rooted phylogenetic analysis of the larger rDNA dataset strongly supports *N. spathiger* and *N. helvetianus* as most closely related among the sampled species and also yields strong support for a clad consisting of these 2 species and *N. filicollis*. However, the outgroup-rooted trees depict the *N. battus* sequences as the sister group to (*N. helvetianus, spathiger, filicollis*), rather than a basal lineage as in similarity analysis, indicating that midpoint rooting may result in an erroneous topology.

The phylogenetic relationships among these 4 species are very strongly supported as assessed by bootstrap resampling in both MP and NJ analyses, and ITS data appear promising for inferring relationships among other species of *Nematodirus*. This phylogenetic hypothesis differs from that inferred from cladistic analysis of morphological data (Hoberg, 1997), where in the same species are related as (((*N. battus, N. helvetianus*), *N. spathiger*), *N. filicollis* = root). This difference has implications for inferring the history of host–parasite diversification; however, the conflict between these 2 phylogenetic hypotheses is due only to differences in the position of the root. Given the outgroup rooting of the sequenced-based tree, the alternative relationships depicted in the morphologically based hypothesis are a significantly worse explanation for these sequence data. With respect to interpreting host associations and diversification, the rDNA tree lacks representatives of species parasitizing cervids and camels that are essential to understanding potential changes in host associations through time. Yet, the topology for the 4 species represented in this rDNA tree is consistent with caprine bovids as ancestral hosts, with a subsequent host-shift to Bovinae in *N. helvetianus*. This interpretation assumes that caprines are the representative host for *N. battus*, but this assumption is not entirely straightforward because this species is capable of infecting certain cervids, boids, and lagomorphs (Hoberg, 1997). The morphological cladogram (Hoberg, 1997) also supports caprine boids as ancestral hosts but indicates that the sister species of *N. battus* utilize Cervidae as hosts, supporting the contention of Jansen (1973) that *N. battus* shares most recent common ancestry with species from cervids.

**Geographic samples of *N. battus***

There is insufficient phylogenetic resolution to either corroborate or refute a hypothesis for the origin and distribution of *N. battus* in North America, including the relationships among isolates derived from the U.K., Canada, and Oregon. The observation that the only identical sequences in the dataset are from Canada (PEI PCR product, PEI cl 1) and the U.S.A. (Oregon cl 2) indicates that some of the sequences from Canada and the U.S.A. are more similar to one another than to other sequences obtained in this study. Despite this similarity, there are no shared derived characters showing ancestor–descendant relationships among these particular sequences. Thus, these identical sequences only reiterate that testing the hypothesis of an introduction to Oregon via Canada requires additional phylogenetically informative data, perhaps using sequences more likely to show phylogeographic patterns for nematodes such as mitochondrial DNA (Blouin et al., 1995). However, these samples of *N. battus* sequences do show differences in amounts of ITS variation, with Oregon and PEI samples having the lowest number of variable sites (1 in each), whereas the other samples have from 6 to 13 variable sites. This reduced variation is consistent with theoretical predictions involving introduction of a limited number of individuals (founder effect) or a subsequent population bottleneck (Nadler, 1995).

Resolution in the strict consensus of MP trees (standard recoded alignment) shows a cladistic relationship among 3 clones, the 2 sequenced from St. Kilda and clone 3 from Norway. Bootstrap resampling shows strong support (in both MP and NJ trees) for a sister-group relationship between 2 of these sequences, Norway cl 3 and St. Kilda cl 8; the NJ tree shows that the branch supporting these taxa is the longest for *N. battus* sequences. The relationship between both St. Kilda sequences and 1 (of 3) Norway sequences is intriguing given hypotheses of host (and parasite) introduction to the St. Kilda archipelago. The sample of *N. battus* from St. Kilda was obtained from Soay sheep residing on the island of Hirta where they had been translocated from the island of Soay in 1932 (Boyd et al., 1964; Campbell, 1974). Soay sheep are a relictual breed with obscure origins but are recognized as the most “primitive sheep” surviving in Europe. The Soay-type of sheep was apparently geographically widespread and has survived in isolation on the island of Soay since the Neolithic (Ryder, 1968). Limited introductions to St. Kilda of 4-horned Hebridean sheep in medieval times and black-faced sheep in the mid-1800s had limited success and neither was established on Soay (Boyd et al., 1964). A controversial hypothesis suggests that the Norse originally brought Soay sheep to the archipelago (Ryder, 1968), and the
relationship between the St. Kilda clones and 1 contemporary sequence from Norway may reflect this scenario. Also, interestingly, Soay sheep were established at several locations in the Borderlands of Scotland in the late 1800s and early 1900s (Elwes, 1912), in the region where *N. battus* was first discovered and became prominent in the 1950s. Based on these ITS data, there is no indication of a sister-group relationship between the St. Kilda and British samples. Unfortunately, definitive documentation of *N. battus* on St. Kilda is very recent (Gulland, 1992; Gulland and Fox, 1992), although an earlier report suggests that it may have been observed previously (Cheyne et al., 1974). Also, given the potential for more recent introductions of *N. battus* from the mainland via transport of other sheep breeds, it would seem unlikely that only sequences representing an early translocation and establishment would be found if additional sampling was performed on the archipelago.

Alternatively, the occurrence of *N. battus* in Soay sheep, which represent the original domesticated stock from the Neolithic in Europe, may indicate that the parasite is considerably more widespread in the western Palearctic. The recognition of relicual or otherwise isolated populations of *N. battus* in Yugoslavia and Italy (Lepojev, 1963; Nardi et al., 1974) may be consistent with this distribution history. It is clear that the history for *N. battus* in the Palearctic is complex and remains to be evaluated fully. Within a molecular phylogenetic context, exploring this history requires examination and reconciliation with more recent reports of *N. battus* from Britain, Norway, France, the Netherlands, and Denmark (Hoberg, 1997).

The only other cladistic structure for *N. battus* sequences involves monophyly of the sequences exclusive of Weybridge cl 5, but this is weakly supported by bootstrap MP resampling and, therefore, must be interpreted cautiously. In addition, this relationship is not recovered in the consensus of NJ trees (standard versus truncated alignments), indicating that this relationship is sensitive to alignment ambiguity. The most conservative interpretation of relationships among *N. battus* sequences is that there is little phylogenetic structure supporting ancestor–descendant relationships. This lack of resolution likely results from lack of sufficient time for rDNA repeat homogenization within geographic localities such that much of the observed variation is not partitioned among localities. It is also possible that the effect of recent translocations of hosts and establishment of their parasites may have confounded previously established patterns. Discriminating between these and other alternatives may be aided by studying genetic markers that do not typically recombine or undergo concerted evolution, such as mitochondrial DNA.

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