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Relationships of *Nematodirus* Species and *Nematodirus battus* Isolates (Nematoda: Trichostrongyloidea) Based on Nuclear Ribosomal DNA Sequences

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RELATIONSHIPS OF *NEMATODIRUS* SPECIES AND *NEMATODIRUS 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 Bovinae 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 intraindividual 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 Pilitt, 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 ultracold 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

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1986–1988 (see Rickard et al., 1989, 1997) as follows: (1) *N. filicollis,* USNPC 86988, adult male and female specimens; (2) *N. spathiger,* USNPC 86989, adult male and female specimens; and (3) from sheep and cattle, *Bos taurus* L., adult male and female specimens of *N. helvetianus,* USNPC 86990. Specimens of *N. battus,* both adult males and females, representing putative discrete populations were as follows: Oregon, USNPC 86987, in naturally infected *O. aries* from the Willamette Valley, adjacent to Corvallis, Oregon, in 1986–1988 by E. P. Hoberg and L. G. Rickard (see Rickard et al., 1997); Prince Edward Island (PEI), Canada, USNPC 70248, derived from single passage in experimental *O. aries* at BNPCU on 20 December 1990, based on a pooled sample of eggs in feces collected from 2 naturally infected lambs at Montague, PEI, in July and August 1990 by E.P.H.; Weybridge, United Kingdom, USNPC 70296 and 84038, derived from single passage in 2 experimental *O. aries* at the BNPCU on 30 April 1991, based on L3 hatched from eggs from the Weybridge strain in March 1991 by M. B. Lancaster; Norway, USNPC 84040, derived from single passage in 2 experimental *O. aries* at BNPCU on 24 and 25 October 1991, based on a pooled sample of eggs in feces collected from 50 naturally infected lambs at Ullevalsveinen, Norway in July 1991 by O. Helle; St. Kilda, United Kingdom, USNPC 84039, derived from single passage in experimental *O. aries* at BNPCU on 21 April 1992, based on a pooled sample of eggs in feces collected from Soay sheep, *O. aries,* from West Meadow no. 3 on the Island of Hirta, St. Kilda Archipelago (see Gulland and Fox, 1992) in August and September 1991 by F. M. D. Gulland. The Weybridge strain had its source in Northumberland, northern England in 1955 (see Gibson, 1959); since the 1970s, no new genetic material has been intentionally introduced to this strain that is now maintained at the Central Veterinary Laboratory, Surrey U.K. (K. R. Hunt, pers. comm.).

Protocols for the culture of eggs and larvae of *N. battus* primarily followed those outlined by the Ministry of Agriculture, Fisheries and Food (MAFF, 1984). Eggs were recovered from emulsified feces using sieves of 44-μm mesh; flotations were done in saturated NaCl, sugar, or $ZnSO_4$. Cultures were maintained in 0.5% potassium dichromate and incubated for approximately 6 wk at 20 C. Infective larvae were administered to parasite-free lambs (raised in a flock at BNPCU) at the age of weaning; the infective dose varied according to the number of L3 available following incubation and release of larvae from cultures representing each of the populations.

At necropsy, adult nematodes were collected from the anterior third of the small intestine of each lamb, washed in saline, and recovered using sieves of 44-μm mesh. To eliminate the potential for cross-contamination, all facilities and instruments were disinfected, autoclaved, or steam-cleaned between respective experimental infections at the BNPCU. The identity of individual worms was confirmed by microscopy, with reference to standard diagnostic characters previously established for *N. battus* and other species (Crofton and Thomas, 1951, 1954; Lichtenfels and Pilitt, 1983; Hoberg et al., 1986). Specimens were separated by sex and frozen at −70 C in lots of 10 or more. *Uncinaria stenocephala* was collected from an arctic fox (*Alopex lagopus*), St. Paul Island, Alaska, by G. Lyons.

DNA amplification and sequencing

Nucleic acids were extracted from frozen (−70 C) tissue samples of pooled adults or individual males (as specified) that had previously been prepared as temporary saline mounts and identified individually by microscopy. For pooled samples, nematodes were macerated in pH 8.0 TE buffer (Ausubel et al., 1989) and digested using Proteinase K (1 μg/μl final concentration; Sigma, St. Louis, Missouri) at 50 C until only the cuticle remained. Nucleic acids were obtained from the supernatant using phenol–chloroform enrichment, ethanol/ammonium acetate precipitation (Ausubel et al., 1989). The resulting pellet was washed with 70% ethanol, resuspended in TE buffer (pH 8.0), treated with 50 μg of RNAse A (1 hr at 37 C), and DNA recovered following reprecipitation with ethanol. Nucleic acids in extracts were quantified by spectrophotometry. For individual *Nematodirus* specimens, nucleic acids were obtained using a DNA binding (''glass milk'') method employing isothiocyanate and guanidinium (ID Pure Genomic DNA Kit, ID Labs Biotechnology; London, Ontario, Canada). Nucleic acids were isolated from an individual female *U. stenocephala* using this DNA binding method. Nucleic acids obtained from individual nematodes were not quantified prior to use in polymerase chain reaction (PCR) amplifications.

A region of nuclear rDNA, including the 18S 3′ end, ITS-1, ITS-2, 5.8S subunit, and 28S 5′ end, was amplified by PCR. Amplification 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 3′ end (primer no. 93, 2635–2653 5′-TTGAACCGGGTAAAAGTCG) and 28S 5′ end (primer no. 94, 3745–3764 5′-TTAGTTTCTTTTCCTCCGCT) 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 $MgCl_2$ 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 mM EDTA), and reconstituted to 48 μl by vacuum evaporation. Seven and one-half microliters 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 ml Luria–Bertani broth cultures. Two clones were sequenced for each species and geographic isolate of *Nematodirus*. Clones were sequenced manually using the Delta-Taq cycle sequencing kit (Amersham, Piscataway, New Jersey) and α^{32}P-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′-GGCCAGTGAATTGTAATACGACTC; primer no. 157, pGEM-T 110–135 5′-GACACTATAGAATACTCAAGCTATGC), and 4 internal rDNA primers (no. 656, 5′-GCTGTCAGGTAGTCTTAATGATCCG, no. 389, 5′-TGCAGACGCTTAGAGTGGTG, no. 264, 5′-CGTTTTTCATCGATACGCG, no. 655, 5′-GTTTACAACCCTGAACCAGACGT) based on sequence data obtained using vector primers. Three of the 4 internal primers annealed to regions that were not invariant among the *Nematodirus* taxa. To test for intraindividual rDNA variation, PCR product amplified from DNA of individual males was sequenced directly. PCR amplifications from individual males were performed using a proofreading DNA polymerase (ID Proof, ID Labs Biotechnology); amplification and cycling conditions were as previously described except a 58 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 sequencing chemistry and an ABI 377 DNA Sequencer. As 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

Invariant 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* (Ancylostomatoidea) and *C. elegans* (Rhabditoidea). 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) with 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 representing 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 repeatable 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-nt 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, CI 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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                                1                   21                  41
N. battus Norway PCR            TCGAAACCTT CACGGGTTCG TTTGATCGCC TCGAGAAACC AACCGACTGG CATGTTTGCG
N. battus Norway cl 2           TCGAAACCTT CACGGGTTCG TTTGATCGCC TCGAGAAACC AACCGACTGG CATGTTTGCG
N. battus Norway cl 3           TCGAAACCTT CACGGGTTCG TTTGATCGCC TCGAGAAACC AACCGACTGG CATGTTTGCG
N. battus St. Kilda cl 8        TCGAAACCTT CACGGGTTCG TTTGATCGCC TCGAGAAACC AACCGACTGG CATGTTTGCG
N. battus St. Kilda cl 9        TCGAAACCTT CACGGGTTCG TTTGATCGCC TCGAGAAACC AACCGACTGG CATGTTTGCG
N. battus Oregon PCR            TCGAAACCTT CACGGGTTCG TTTGATCGCC TCGAGAAACC AACCGACTGG CATGTTTGCG
N. battus Oregon cl 1           TCGAAACCTT CACGGGTTCG TTTGATCGCC TCGAGAAACC AACCGACTGG CATGTTTGCG
N. battus Oregon cl 2†          TCGAAACCTT CACGGGTTCG TTTGATCGCC TCGAGAAACC AACCGACTGG CATGTTTGCG
N. battus Weybridge PCR         TCGAAACCTT CACGGGTTCG TTTGATCGCC TCGAGAAACC AACCGACTGG CATGTTTGCG
N. battus Weybridge cl 4        TCGAAACCTT CACGGGTTCG TTTGATCGCC TCGAGAAACC AACCGACTGG CATGTTTGCG
N. battus Weybridge cl 5        TCGAAACCTT CACGGGTTCG TTTGATCGCC TCGAGAAACC AACCGATTGG CATGTTTGCG
N. helvetianus PCR              TCGAAACCTT CACGGGTTCG TTTGATCATC ACGAGAAACC AACCGCCTGG TATGTTTACG
N. helvetianus cl 1             TCGAAACCTT CACGGGTTCG TTTGATCATC ACGAGAAACC AACCGCCTGG TATGTTTACG
N. helvetianus cl 4             TCGAAACCTT CACGGGTTCG TTTGATCATC ACGAGAAACC AACCGCCTGG TATGTTTACG
N. spathiger PCR                TCGAAACCTT CACGGGTTCG TYTGATCATC ACGAGAAACC AACCGTCYGG TATGTTTACG
N. spathiger cl 4               TCGAAACCTT CACGGGTTCG TCTGATCATC ACGAGAAACC AACCGTCCGG TATGTTTACG
N. spathiger cl 5               TCGAAACCTT CACGGGTTCG TCTGATCATC ACGAGAAACC AACCGTCCGG TATGTTTACG
N. filicollis cl 2              TCGAAACCTT CACGGGTTCG CTTGATCTTG ACGAGAAACC AACCGACCGG CATGTTTGCG
N. filicollis cl 4              TCGAAACCTT CACGGGTTCG CTTGATCTTG ACGAGAAACC AACCGACCGG CATGTTTGCG
                                ********** **********   *****     ********* *****   **  ****** **

                                61                  81                  101
N. battus Norway PCR            GCCATGYCGC GAACGTTGGG AGTATCACCC CCGTTGGAGC TCTATGGAAG GTGTCTACCG
N. battus Norway cl 2           GCCATGTCGC GAACGTTGGG AGTATCACCC CCGTTGGAGC TCTATGGAAG GTGTCTACCG
N. battus Norway cl 3           GCCATGTCGC GAACGTTGGG AGTATCACCC CCGTTGGAGC TCTATGGAAG GTGTCTACCG
N. battus St. Kilda cl 8        GCCATGCCGC GAGCGTTGGG AGTATCACCC CCGTTGGGGC TCTATGGAAG GTGTCTACCG
N. battus St. Kilda cl 9        GCCATGCCGC GAACGTTGGG AGTATCACCC CCGTTGGAGC TCTATGGAAG GTGTCTACCG
N. battus Oregon PCR            GCCATGCCGC GAACGTTGGG AGTATCACCC CCGTTGGAGC TCTATGGAAG GTGTCTACCG
N. battus Oregon cl 1           GCCATGCCGC GAACGTTGGG AGTATCACCC CCGTTGGAGC TCTATGGAAG GTGTCTACCG
N. battus Oregon cl 2†          GCCATGTCGC GAACGTTGGG AGTATCACCC CCGTTGGAGC TCTATGGAAG GTGTCTACCG
N. battus Weybridge PCR         GCCATGYCGC GAACGTTGGG AGTATCACCC CCGTTGGAGC TCTATGGAAG GTGTCTACCG
N. battus Weybridge cl 4        GCCATGCCGC GAACGTTGGG AGTATCACCC CCGTTGGAGC TCTATGGAAG GTGTCTACCG
N. battus Weybridge cl 5        GCCATGTCGC GAACGTTGGG AGTATCACCC CCGTTGGGGC TCTATGGAAG GTGTCTACCG
N. helvetianus PCR              ACTTTGTCGT GAACGTTGGT AGTATCACCC CCGTTGGAGC TCTATGTGAG GTGTCTACCG
N. helvetianus cl 1             ACTTTGTCGT GAACGTTGGT AGTATCACCC CCGTTGGAGC TCTATGTGAG GTGTCTACCG
N. helvetianus cl 4             ACTTTGTCGT GAACGTTGGT AGTATCACCC CCGTTGGAGC TCTATGTGAG GTGTCTACCG
N. spathiger PCR                ACTTTGTCGT GAACGTTGGT AGTATCACCC CCGTTGGAGC TCTCYGTGAG GTGTCTACCG
N. spathiger cl 4               ACTTTGTCGT GAACGTTGGT AGTATCACCC CCGTTGGAGC TCTCCGTGAG GTGTCTACCG
N. spathiger cl 5               ACTTTGTCGT GAACGTTGGT AGTATCACCC CCGTTGGAGC TCTCCGTGAG GTGTCTACCG
N. filicollis cl 2              ACATTGTCGT AAACGTTGGG AGTATCACCC CCGTTGGAGC TCTATGTGAG GTGTCTACCG
N. filicollis cl 4              ACATTGTCGT AAACGTTGGG AGTATCACCC CCGTTGGAGC TCTATGTGAG GTGTCTACCG
                                 *  ** **   ********  ********** ******* ** ***  *  ** **********

                                121                 141                 161
N. battus Norway PCR            TACGCGATGA GTCGTTCGTG AGTGGCGGCA GTGAATCGTC GTGCAAAGTT CCCACCTACG
N. battus Norway cl 2           TACGCGATGA GTCGTTCGTG AGTGGCGGCA GTGAATCGTC GTGCAAAGTT CCCACCTACG
N. battus Norway cl 3           TACGCGATGA GTCGTTCGTG AGTGGCGGCA GTGAATCGTC GTGCAAAGTT CCCACCTACG
N. battus St. Kilda cl 8        TACGCGATGA GTCGTTCGTG AGTGGCGGCA GTGAATCGTC GTGCAAAGTT CCCACCTACG
N. battus St. Kilda cl 9        TACGCGATGA GTCGTTCGTG AGTGGCGGCA GTGAATCGTC GTGCAAAGTT CCCACCTACG
N. battus Oregon PCR            TACGCGATGA GTCGTTCGTG AGTGGCGGCA GTGAATCGTC GTGCAAAGTT CCCACCTACG
N. battus Oregon cl 1           TACGCGATGA GTCGTTCGTG AGTGGCGGCA GTGAATCGTC GTGCAAAGTT CCCACCTACG
N. battus Oregon cl 2†          TACGCGATGA GTCGTTCGTG AGTGGCGGCA GTGAATCGTC GTGCAAAGTT CCCACCTACG
N. battus Weybridge PCR         TACGCGATGA GTCGTTCGTG AGTGGCGGCA GTGAATCGTC GTGCAAAGTT CCCACCTACG
N. battus Weybridge cl 4        TACGCGATGA GTCGTTCGTG AGTGGCGGCA GTGAATCGTC GTGCAAAGTT CCCACCTACG
N. battus Weybridge cl 5        TACGCGATGA GTCGTTCGTG AGTGGCGGCA GTGAATCGTC GTGCAAAGTT CCCACCTACG
N. helvetianus PCR              TACGCGATGA GTCGTTCTTG AGTGGCGGCA GTGATTCGTC GTACAAAGTT CCCATCTAGC
N. helvetianus cl 1             TACGCGATGA GTCGTTCTTG AGTGGCGGCA GTGATTCGTC GTACAAAGTT CCCATCTAGC
N. helvetianus cl 4             TACGCGATGA GTCGTTCTTG AGTGGCGGCA GTGATTCGTC GTACAAAGTT CCCATCTAGC
N. spathiger PCR                TACGCGATGA GTCGTTCTTG AGTGGCGGCA GTGAKTCGTC GTACAAAGTT CCCATCTTGC
N. spathiger cl 4               TACGCGATGA GTCGTTCTTG AGTGGCGGCA GTGATTCGTC GTACAAAGTT CCCATCTTGC
N. spathiger cl 5               TACGCGATGA GTCGTTCTTG AGTGGCGGCA GTGATTCGTC GTACAAAGTT CCCATCTTGC
N. filicollis cl 2              TACGCGATGA GTCGTTCTTG AGTGGCGGCC GTGATTCGTC GTACAAAGTT CCCACATAAA
N. filicollis cl 4              TACGCGATGA GTCGTTCTTG AGTGGCGGCC GTGATTCGTC GTACAAAGTT CCCACATAAA
                                ********** ******* ** *********  **** ***** ** ******* ****  *
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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 = G or T, M = A or C, R = A or G, W = A or T, Y = T or C). Complete alignment with *U. stenocephala* and *C. elegans,* including 18S, 28S, and 5.8S sequences is deposited in TreeBase.

```
                                 181                 201                 221
N. battus Norway PCR             GTGGTCGAGC TTATTAGACT TAATGAGTAT TGCTGGAATG CCGCCTTACT GTTTTACGTT
N. battus Norway cl 2            GTGGTCGAGC TTATTAGACT TAATGAGTAT TGCTGGAATG CCGCCTTACT GTTTTACGTT
N. battus Norway cl 3            GTGGTCGAGC TTATTAGACT TAATGAGTAT TGCTGGAATG CCGCCTTACT GTTTTACGTT
N. battus St. Kilda cl 8         GTGGTCGAGC TTATTAGACT TAATGAGTAT TGCTGGAATG CCGCCTTACT GTTTTACGTT
N. battus St. Kilda cl 9         GTGGTCGAGC TTATTAGACT TAATGAGTAT TGCTGGAATG CCGCCTTACT GTTTTACGTT
N. battus Oregon PCR             GTGGTCGAGC TTATTAGACT TAATGAGTAT TGCTGGAATG CCGCCTTACT GTTTTACGTT
N. battus Oregon cl 1            GTGGTCGAGC TTATTAGACT TAATGAGTAT TGCTGGAATG CCGCCTTACT GTTTTACGTT
N. battus Oregon cl 2†           GTGGTCGAGC TTATTAGACT TAATGAGTAT TGCTGGAATG CCGCCTTACT GTTTTACGTT
N. battus Weybridge PCR          GTGGTCGAGC TTATTAGACT TAATGAGTAT TGCTGGAATG CCGCCTTACT GTTTTACGTT
N. battus Weybridge cl 4         GTGGTCGAGC TTATTAGACT TAATGAGTAT TGCTGGAATG CCGCCTTACT GTTTTACGTT
N. battus Weybridge cl 5         GTGGTCGAGC TTATTAGACT TAATGAGTAT TGCTGGAATG CCGCCTTACT GTTTTACGTT
N. helvetianus PCR               GTGGTCGAGC TTACTAGACT TGATGAGCGT TGCTGGAACG CCGCCTCAAT GTTTTACGTT
N. helvetianus cl 1              GTGGTCGAGC TTACTAGACT TGATGAGCGT TGCTGGAACG CCGCCTCAAT GTTTTACGTT
N. helvetianus cl 4              GTGGTCGAGC TTACTAGACT TGATGAGCGT TGCTGGAACG CCGCCTCAAT GTTTTACGTT
N. spathiger PCR                 ATGGTCGAGC TTACTAGACT TGATGAGCGT TGCTKGAACG CCGCCTCAAT GTTTTACGTT
N. spathiger cl 4                ATGGTCGAGC TTACTAGACT TGATGAGCGT TGCTTGAACG CCGCCTCAAT GTTTTACGTT
N. spathiger cl 5                ATGGTCGAGC TTACTAGACT TGATGAGCGT TGCTGGAACG CCGCCTCAAT GTTTTACGTT
N. filicollis cl 2               GTGGTCGAGC TTGCTAGACT TGATGAGCAT TGCTGGAATG CCGCCTCGCT GTTTTACGTT
N. filicollis cl 4               GTGGTCGAGC TTGCTAGACT TGATGAGCAT TGCTGGAATG CCGCCTCGCT GTTTTACGTT
                                 ********* **  ****** * *****  * **** *** * ******   * **********

                                 241                 261                 281
N. battus Norway PCR             GGTGACTAGG CACAATGCTA GTACCGTGTG GAACCTGGCT GTCAGGTAGT CTTAATGATC
N. battus Norway cl 2            GGTGACTAGG CACAATGCTA GTACCGTGTG GAACCTGGCT GTCAGGTAGT CTTAATGATC
N. battus Norway cl 3            GGTGACTAGG CACAATGCTA GTACCGTGTG GAACCTGGCT GTCAGGTAGT CTTAATGATC
N. battus St. Kilda cl 8         GGTGACTAGG CACAATGCTA GTACCGTGTG GAACCTGGCT GTCAGGTAGT CTTAATGATC
N. battus St. Kilda cl 9         GGTGACTAGG CACAATGCTA GTACCGTGTG GAACCTGGCT GTCAGGTAGT CTTAATGATC
N. battus Oregon PCR             GGTGACTAGG CACAATGCTA GTACCGTGTG GAACCTGGCT GTCAGGTAGT CTTAATGATC
N. battus Oregon cl 1            GGTGACTAGG CACAATGCTA GTACCGTGTG GAACCTGGCT GTCAGGTAGT CTTAATGATC
N. battus Oregon cl 2†           GGTGACTAGG CACAATGCTA GTACCGTGTG GAACCTGGCT GTCAGGTAGT CTTAATGATC
N. battus Weybridge PCR          GGTGACTAGG CACAATGCTA GTACCGTGTG GAACCTGGCT GTCAGGTAGT CTTAATGATC
N. battus Weybridge cl 4         GGTGACTAGG CACAATGCTA GTACCGTGTG GAACCTGGCT GTCAGGTAGT CTTAATGATC
N. battus Weybridge cl 5         GGTGACTAGG CACGATGCTA GTACCGTGTG GAACCTGGCT GTCAGGTAGT CTTAATGATC
N. helvetianus PCR               GGTGATTGTG CACAATGCTG CCACCGTGCG GAACCTGGCT GTCAGGAAGT CTTAATGATC
N. helvetianus cl 1              GGTGATTGTG CACAATGCTG CCACCGTGCG GAACCTGGCT GTCAGGAAGT CTTAATGATC
N. helvetianus cl 4              GGTGATTGTG CACAATGCTG CCACCGTGCG GAACCTGGCT GTCAGGAAGT CTTAATGATC
N. spathiger PCR                 GGTGATTGTG CACAATGCGA CCACCGTGCG GAACCTGGCT GTCAGGAAGT CTTAATGATC
N. spathiger cl 4                GGTGATTGTG CACAATGCGA CCACCGTGCG GAACCTGGCT GTCAGGAAGT CTTAATGATC
N. spathiger cl 5                GGTGATTGTG CACAATGCGA CCACCGTGCG GAACCTGGCT GTCAGGAAGT CTTAATGATC
N. filicollis cl 2               GGTGATTGAG CACAAAGCGA CCATCGTGCG TAACCTGGCT GTCAGGAAAT CTTAATGATC
N. filicollis cl 4               GGTGATTGAG CACAAAGCGA CCATCGTGCG TAACCTGGCT GTCAGGAAAT CTTAATGATC
                                 ***** *  * *** * **     * **** *  ********* ****** * * **********

                                 301                 321                 341
N. battus Norway PCR             CGACACACTT ----GGGCGC CAAAGTAGAA CTCACA-CTT TTTCAATTTG AACGTTTGCA
N. battus Norway cl 2            CGACACACTT ----GGGCGC CAAAGTAGAA CTCACA-CTT TTTCAATTTG AACGTTTGCA
N. battus Norway cl 3            CGACACACTC GGGCGGGCGC CAAAGTAGAA CTCACA-CTT TTTCAATTTG AACGTTTGCA
N. battus St. Kilda cl 8         CGACACACTC GGGCGGGCGC CAAAGTAGAA CTCACA-CTT TTTCAATTTG AACGTTTGCA
N. battus St. Kilda cl 9         CGACACACTC GGGCGGGCGC CAAAGTAGAA CTCACA-CTT TTTCAATTTG AACGTTTGCA
N. battus Oregon PCR             CGACACWCTT ----GGGCGC CAAAGTAGAA CTCACA-CTT TTTCAATTTG AACGTTTGCA
N. battus Oregon cl 1            CGACACACTT ----GGGCGC CAAAGTAGAA CTCACA-CTT TTTCAATTTG AACGTTTGCA
N. battus Oregon cl 2†           CGACACACTT ----GGGCGC CAAAGTAGAA CTCACA-CTT TTTCAATTTG AACGTTTGCA
N. battus Weybridge PCR          CGACACWCTT ----GGGCGC CAAAGTAGAA CTCACA-CTT TTTCAATTTG AACGTTTGCA
N. battus Weybridge cl 4         CGACACTCTT ----GGGCGC CAAAGTAGAA CTCACA-CTT TTTCAATTTG AACGTTTGCA
N. battus Weybridge cl 5         CGACACACTT ----GGGCGC CAAAGTAGAA CTCACA-CTT TTTCAATTTG AACGTTTGCA
N. helvetianus PCR               CGGGCCACCC ----GGGCGC CAACGTAGAA CTCACAAYCT TTCTAATTTG AATATTGGCA
N. helvetianus cl 1              CGGGCCACCC ----GGGCGC CAACGTAGAA CTCACAACCT TTCTAATTTG AATATTGGCA
N. helvetianus cl 4              CGGGCCACCC ----GGGCGC CAACGTAGAA CTCACAATCT TTCTAATTTG AATATTGGCA
N. spathiger PCR                 CGGGTCACCC ----GGRCGC CAACGTAGAA CTCACAATMT TTCYATTTTG AATGTTGGCA
N. spathiger cl 4                CGGGTCACCC ----GGACGC CAACGTAGAA TTCACAATCT TTCTATTTTG AATGTTGGCA
N. spathiger cl 5                CGGGTCACCC ----GGACGC CAACGTAGAA CTCACAATCT TTCTATTTTG AATGTTGGCA
N. filicollis cl 2               CGGCACACCC ----GGACGC CAACGTAAAA CTAACAACTT TTCTATTTTG AATTTTGACA
N. filicollis cl 4               CGGCACACCC ----GGACGC CAACGTAAAA CTAACAACTT TTCTATTTTG AATTTTGACA
                                 **   * *       ** *** *** *** **  * ***   * **  * **** **  **  **
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FIGURE 1. Continued.

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. filicollis,* 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. filicollis* = 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.

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                                361                 381                 401
N. battus Norway PCR            GAATAGTGAC TTTCATGTCA CAATCATGAA AAACGAAGTA CTACAGTATG TCTAGTTATG
N. battus Norway cl 2           GAATAGTGAC TTTCATGTCA CAATCATGAA AAACGAAGTA CTACAGTATG TCTAGTTATG
N. battus Norway cl 3           GAATAGTGAC TTTCATGTCA CAATCATGAA AAACGAAGTA CTACGGTATG TCTAGTTATG
N. battus St. Kilda cl 8        GAATAGTGAC TTTCATGTCA CAATCATGAA AAACGAAGTA CTACAGTATG TCTAGTTATG
N. battus St. Kilda cl 9        GAATAGTGAC TTTCATGTCA CAATCATGAA AAACGAAGTA CTACAGTATG TCTAGTTATG
N. battus Oregon PCR            GAATAGTGAC TTTCATGTCA CAATCATGAA AAACGAAGTA CTACAGTATG TCTAGTTATG
N. battus Oregon cl 1           GAATAGTGAC TTTCATGTCA CAATCATGAA AAACGAAGTA CTACAGTATG TCTAGTTATG
N. battus Oregon cl 2†          GAATAGTGAC TTTCATGTCA CAATCATGAA AAACGAAGTA CTACAGTATG TCTAGTTATG
N. battus Weybridge PCR         GAATAGTGAC TTTCATGTCA CAATCATGAA AAACGAAGTA CTACAGTATG TCTAGTTATG
N. battus Weybridge cl 4        GAATAGTGAC TTTCATGTCA CAATCATGAA AAACGAAGTA CTACAGTATG TCTAGTTATG
N. battus Weybridge cl 5        GAGTAGTGAC TTTCATGTCA CAATCATGAA AAACGAAGTA CTACAGTATG TCTAGTTATG
N. helvetianus PCR              GAATCGTGAT CGATG-ATCA CTCT-ATGAA GAACGATAGA ATACAGCATG GATTGTTTTG
N. helvetianus cl 1             GAATCGTGAT CGATG-ATCA CTCT-ATGAA GAACGATAGA ATACAGCATG GATTGTTTTG
N. helvetianus cl 4             GAATCGTGAT CGATG-ATCA CTCT-ATGAA GAACGATAGA ATACAGCATG GATTGTTTTG
N. spathiger PCR                GAATCGTGAT CKATG-ATCA CTCT-ATGAA GAACGATATA ATACAGCATG GATTGTTTTG
N. spathiger cl 4               GAATCGTGAT CGATG-ATCA CTCT-ATGAA GAACGATATA ATACAGCATG GATTGTTTTG
N. spathiger cl 5               GAATCGTGAT CGATG-ATCA CTCT-ATGGA GAACGATATA ATACAGCATG GATTGTTTTG
N. filicollis cl 2              GA-CCGTGAT CGATGTATCA CTAT-ACGAA AAACGATATA TTACAGCATG GATTGTTGTG
N. filicollis cl 4              GA-CCGTGAT CGATGTATCA CTAT-ACGAA AAACGATATA TTACAGCATG GATTGTTGTG
                                **   ****         *** *  * * * *  *****   *  *** * ***   * *** **

                                421                 441                 461
N. battus Norway PCR            CTGTATGTCG AATGGTACTA TC----ACAT AGTCCCGGTA TA-CCCATTC AAGTAAGGAA
N. battus Norway cl 2           CTGTATGTCG AATGGTACTA TC----GCAT AGTCCCGGTA TA-CCCATTC AAGTAAGGAA
N. battus Norway cl 3           CTGTATGTCG AATGGTACTA TCC---GCAT AATCCCGGTT TACCCCATTC AAGTAAAGAA
N. battus St. Kilda cl 8        CTGTATGTCG AATGGTACTA TCC---GCAT AATCCCGGTT TACCCCATTC AAGTAAAGAA
N. battus St. Kilda cl 9        CTGTATGTCG AATGGTACTA TC----ACAT AGTCCCGGTA TA-CCCATTC AAGTAAGGAA
N. battus Oregon PCR            CTGTATGTCG AATGGTACTA TC----ACAT AGTCCCGGTA TA-CCCATTC AAGTAAGGAA
N. battus Oregon cl 1           CTGTATGTCG AATGGTACTA TC----ACAT AGTCCCGGTA TA-CCCATTC AAGTAAGGAA
N. battus Oregon cl 2†          CTGTATGTCG AATGGTACTA TC----ACAT AGTCCCGGTA TA-CCCATTC AAGTAAGGAA
N. battus Weybridge PCR         CTGTATGTCG AATGGTACTA TC----ACAT AGTCCCGGTA TA-CCCATTC AAGTAAGGAA
N. battus Weybridge cl 4        CTGTATGTCG AATGGTACTA TC----ACAT AGTCCCGGTA TA-CCCATTC AAGTAAGGAA
N. battus Weybridge cl 5        CTGTATGTCG AATGGTACTA TC----ACAT AGTCTCGGTA TA-CCCATTC AAGTAAGGAA
N. helvetianus PCR              CTGTGTGTCG AGTGGTACTT GCTGT----- -GTTACAGCG AATCCCATTC AAGTGAAGAA
N. helvetianus cl 1             CTGTGTGTCG AGTGGTACTT GCTGT----- -GTTACAGCG AATCCCATTC AAGTGAAGAA
N. helvetianus cl 4             CTGTGTGTCG AGTGGTACTT GCTGT----- -GTTACAGCG AATCCCATTC AAGTGAAGAA
N. spathiger PCR                CTGTGTGTCG AGTGGKACTT GCTGT----- -GTTACAGCG AATCCCATTC AAGTGAAGAA
N. spathiger cl 4               CTGTGTGTCG AGTGGTACTT GCTGT----- -GTTACAGCG AATCCCATTC AAGTGAAGAA
N. spathiger cl 5               CTGTGTGTCG AGTGGCACTT GCTGT----- -GTTACAGCG AATCCCATTC AAGTGAAGAA
N. filicollis cl 2              CTGTGTGTCG AATGGTACTT GCTGTTACAT CGTAACAGCC AATCCCATTC AAGTGAAGAA
N. filicollis cl 4              CTGTGTGTCG AATGGTACTT GCTGTTACAT CGTAACAGTC AATCCCATTC AAGTGAAGAA
                                **** ***** * *** ***   *          **  * *    * ******* **** * ***

                                481                 501                 521
N. battus Norway PCR            TGTTTGCAAC ATGGCTTCGT GCTGGTGTCA AAGCCCCTGA ATGATGTGGA CGCGATTGTA
N. battus Norway cl 2           TGTTTGCAAC ATGGCTTCGT GCTGGTGTCA AAGCCCCTGA ATGATGTGGA CGCGATTGTA
N. battus Norway cl 3           TGTTTGCAAC ATGGCTTCGT GCTGGTGTCA GAGCCCCTGA ATGATGTGGA CGCGATTGTA
N. battus St. Kilda cl 8        TGTTTGCAAC ATGGCTTCGT GCTGGTGTCA GAGCCCCTGA ATGATGTGGA CGCGATTGTA
N. battus St. Kilda cl 9        TGTTTGCAAC ATGGCTTCGT GCTGGTGTCA AAGCCCCTGA ATGATGTGGA CGCGATTGTA
N. battus Oregon PCR            TGTTTGCAAC ATGGCTTCGT GCTGGTGTCA AAGCCCCTGA ATGATGTGGA CGCGATTGTA
N. battus Oregon cl 1           TGTTTGCAAC ATGGCTTCGT GCTGGTGTCA AAGCCCCTGA ATGATGTGGA CGCGATTGTA
N. battus Oregon cl 2†          TGTTTGCAAC ATGGCTTCGT GCTGGTGTCA AAGCCCCTGA ATGATGTGGA CGCGATTGTA
N. battus Weybridge PCR         TGTTTGCAAC ATGGCTTCGT GCTGGTGTCA AAGCCCCTGA ATGATGTGGA CGCGATTGTA
N. battus Weybridge cl 4        TGTTTGCAAC ATGGCTTCGT GCTGGTGTCA AAGCCCCTGA ATGATGTGGA CGCGATTGTA
N. battus Weybridge cl 5        TGTTTGCAAC ATGGCTTCGT GCTGGTGTCA AGGCCCCTGA ATGATGTGGA CGCGATTGTA
N. helvetianus PCR              AGTTTGCAAC ATGGCTCTGT ATTAGTGTCA AAGCTCCTGA ATGATGTGAA CGCGATTGTT
N. helvetianus cl 1             AGTTTGCAAC ATGGCTCTGT ATTAGTGTCA AAGCTCCTGA ATGATGTGAA CGCGATTGTT
N. helvetianus cl 4             AGTATGCAAC ATGGCTCTGT ATTAGTGTCA AAGCTCCTGA ATGATGTGAA CGCGATTGTT
N. spathiger PCR                AGTTTGCAAC ATGGCTCTGT ATTAGTGTCA AAGCTCCTGA ATGATGTGAA CGCGATTGTT
N. spathiger cl 4               AGTTTGCAAC ATGGCTCTGT ATTAGTGTCA AAGCTCCTGA ATGATGTGAA CGCGATTGTT
N. spathiger cl 5               AGTTTGCAAC ATGGCTCTGT ATTAGTGTCA AAGCTCCTGA ATGATGTGAA CGCGATTGTT
N. filicollis cl 2              TGTTTGCAAC GTGGCTTTGT GATGGTGTCA AAGCTCCTGA ATGATGTGAA CGCGATTGTT
N. filicollis cl 4              TGTTTGCAAC GTGGCTTTGT GATGGTGTCA AAGCTCCTGA ATGATGTGAA CGCGATTGTT
                                 ** ******  *****  **   * ******   ** ***** ******** * *********
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FIGURE 1. Continued.

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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                                541                   561                   581
N. battus Norway PCR            GCCGTATTGA GTTCTACTCG ATGAGAATGA GATGAATTCA ACGGGGAACT GACTGTAGGC
N. battus Norway cl 2           GCCGTATTGA GTTCTACTCG ATGAGAATGA GATGAATTCA ACGGGGAACT GACTGTAGGC
N. battus Norway cl 3           GCCGTATTGA GTTGTACTCG ATGAGAATGA GATGAATTCA ACGGGGAACT GACTGTAGGC
N. battus St. Kilda cl 8        GCCGTATTGA GTTCTACTCG ATGAGAATGA GATGAATTCA ACGGGGAACT GACTGTAGGC
N. battus St. Kilda cl 9        GCCGTATTGA GTTCTACTCG ATGAGAATGA GATGAATTCA ACGGGGAACT GACTGTAGGC
N. battus Oregon PCR            GCCGTATTGA GTTCTACTCG ATGAGAATGA GATGAATTCA ACGGGGAACT GACTGTAGGC
N. battus Oregon cl 1           GCCGTATTGA GTTCTACTCG ATGAGAATGA GATGAATTCA ACGGGGAACT GACTGTAGGC
N. battus Oregon cl 2†          GCCGTATTGA GTTCTACTCG ATGAGAATGA GATGAATTCA ACGGGGAACT GACTGTAGGC
N. battus Weybridge PCR         GCCGTATTGA GTTCTACTCG ATGAGAATGA GATGAATTCA ACGGGGAACT GACTGTAGGC
N. battus Weybridge cl 4        GCCGTATTGA GTTCTACTCG ATGAGAATGA GATGAATTCA ACGGGGAACT GACTGTAGGC
N. battus Weybridge cl 5        GCCGTATTGA GTTCTACTCG ATGAGAATGA GATGAATTCA ACGGGGAACT GACTGTAGGC
N. helvetianus PCR              GCCGTGTCGA ATCGTACTCG ATGAGAATGA GATGGATTTG ATCGGGGACC TGCTGTGAAC
N. helvetianus cl 1             GCCGTGTCGA ATCGTACTCG ATGAGAATGA GATGGATTTG ATCGGGGACC TGCTGTGAAC
N. helvetianus cl 4             GCCGTGTCGA ATCGTACTCG ATGAGAATGA GATGGATTTG ATCGGGGACC TGCTGTGAAC
N. spathiger PCR                ACCGTGTCGA ATCGTACTCG GTGAGATTGA GATGGATTTG ATCGGGGACC TGCTGTGAAC
N. spathiger cl 4               ACCGTGTCGA ATCGTACTCG GTGAGATTGA GATGGATTTG ATCGGGGACC TGCTGTGAAC
N. spathiger cl 5               ACCGTGTCGA ATCGTACTCG GTGAGATTGA GATGGATTTG ATCGGGGACC TGCTGTGAAC
N. filicollis cl 2              ACCGTATCAA AATGTGCTCG ATGAGTATGA GATGAGTTTG ATCGGGGACC TGCTCTTAAC
N. filicollis cl 4              ACCGTATCAA AATGTGCTCG ATGAGTATGA GATGAGTTTG ATCGGGGACC TGCTCTTAAC
                                 **** *  *     * ****  ****  *** ****  **   *  *** **    ** *   *
                                601                   621
N. battus Norway PCR            AATCGATTGT CCTGACATCA TTTGCAT
N. battus Norway cl 2           AATCGATTGT CCTGACATCA TTTGCAT
N. battus Norway cl 3           AATCGATTGT CCTGACATCA TTTGCAT
N. battus St. Kilda cl 8        AATCGATTGT CCTGACATCA TTTGCAT
N. battus St. Kilda cl 9        AATCGATTGT CCTGACATCA TTTGCAT
N. battus Oregon PCR            AATCGATTGT CCTGACATCA TTTGCAT
N. battus Oregon cl 1           AATCGATTGT CCTGACATCA TTTGCAT
N. battus Oregon cl 2†          AATCGATTGT CCTGACATCA TTTGCAT
N. battus Weybridge PCR         AATCGATTGT CCTGACATCA TTTGCAT
N. battus Weybridge cl 4        AATCGATTGT CCTGACATCA TTTGCAT
N. battus Weybridge cl 5        AATCGATTGT CCTGACATCA TTTGCAT
N. helvetianus PCR              AATCGACTGC TCCGACATCA TTTGCAT
N. helvetianus cl 1             AATCGACTGC TCCGACATCA TTTGCAT
N. helvetianus cl 4             AATCGACTGC TCCGACATCA TTTGCAT
N. spathiger PCR                AATCGATTGT TCTGACATCA TTTGCAT
N. spathiger cl 4               AATCGATTGT TCTGACATCA TTTGCAT
N. spathiger cl 5               AATCGATTGT TCTGACATCA TTTGCAT
N. filicollis cl 2              AATCGATTGT TCAGACATCA TTTGCAT
N. filicollis cl 4              AATCGATTGT TCAGACATCA TTTGCAT
                                ****** **   * ******* *******
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FIGURE 1. Continued.

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

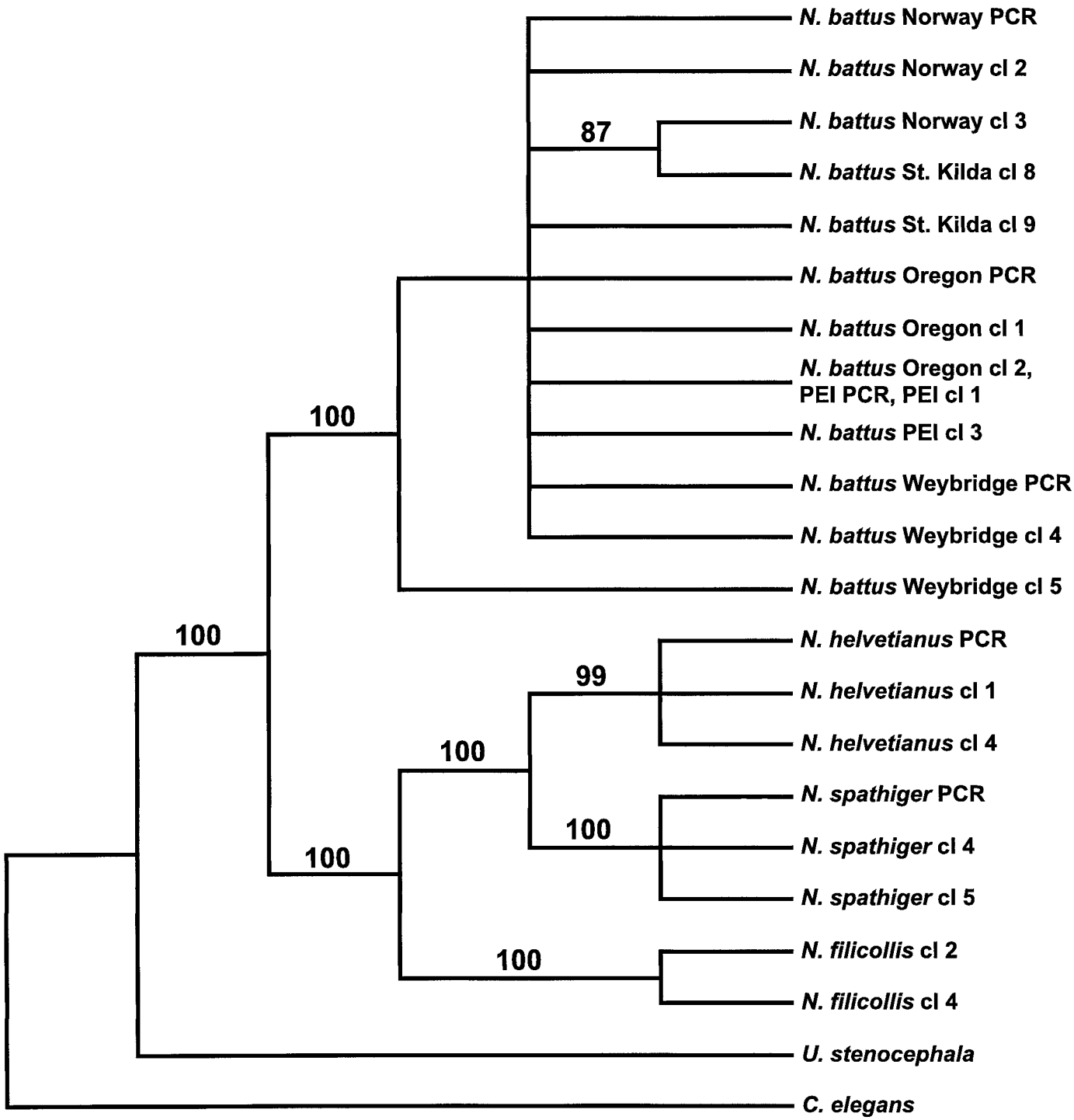

FIGURE 2. Strict consensus of MP trees (671 steps, CI = 0.792, HI = 0.208) based on 194 parsimony-informative characters of the standard alignment with recoded gaps. The bootstrap MP tree (1,000 replicates) was of the same topology; bootstrap percentages of clades >60% are shown above internal nodes. PCR = direct sequence with DNA obtained from a single male specimen; cl = clone of PCR product with DNA obtained from a pool of specimens (mixed sex).

(Arnheim, 1983). 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-

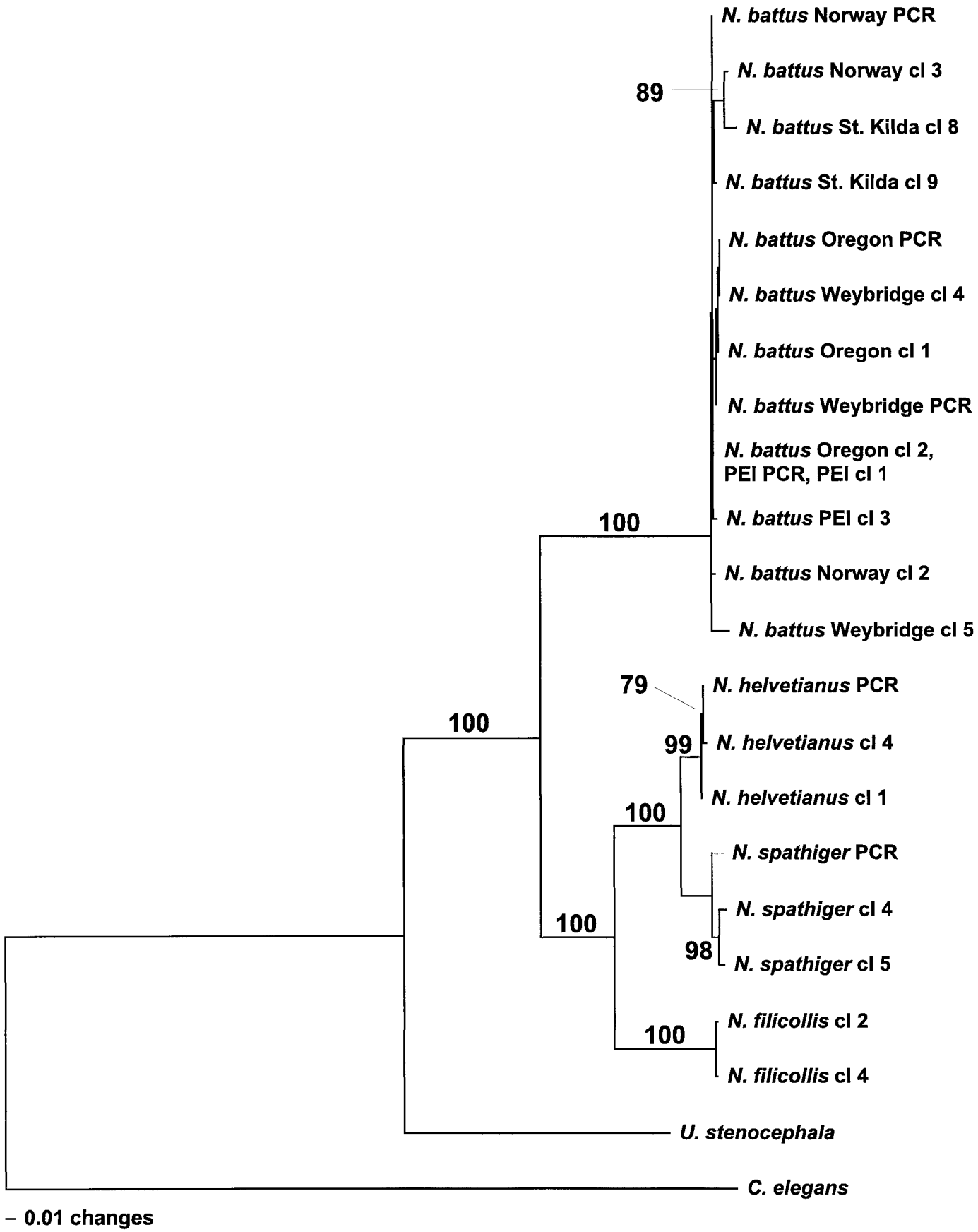

FIGURE 3. Neighbor-joining phylogram based on log-determinant distances. Bootstrap values for NJ inference (1,000 replicates) are shown when >60%. Branch lengths are scaled to expected amount of change per site.

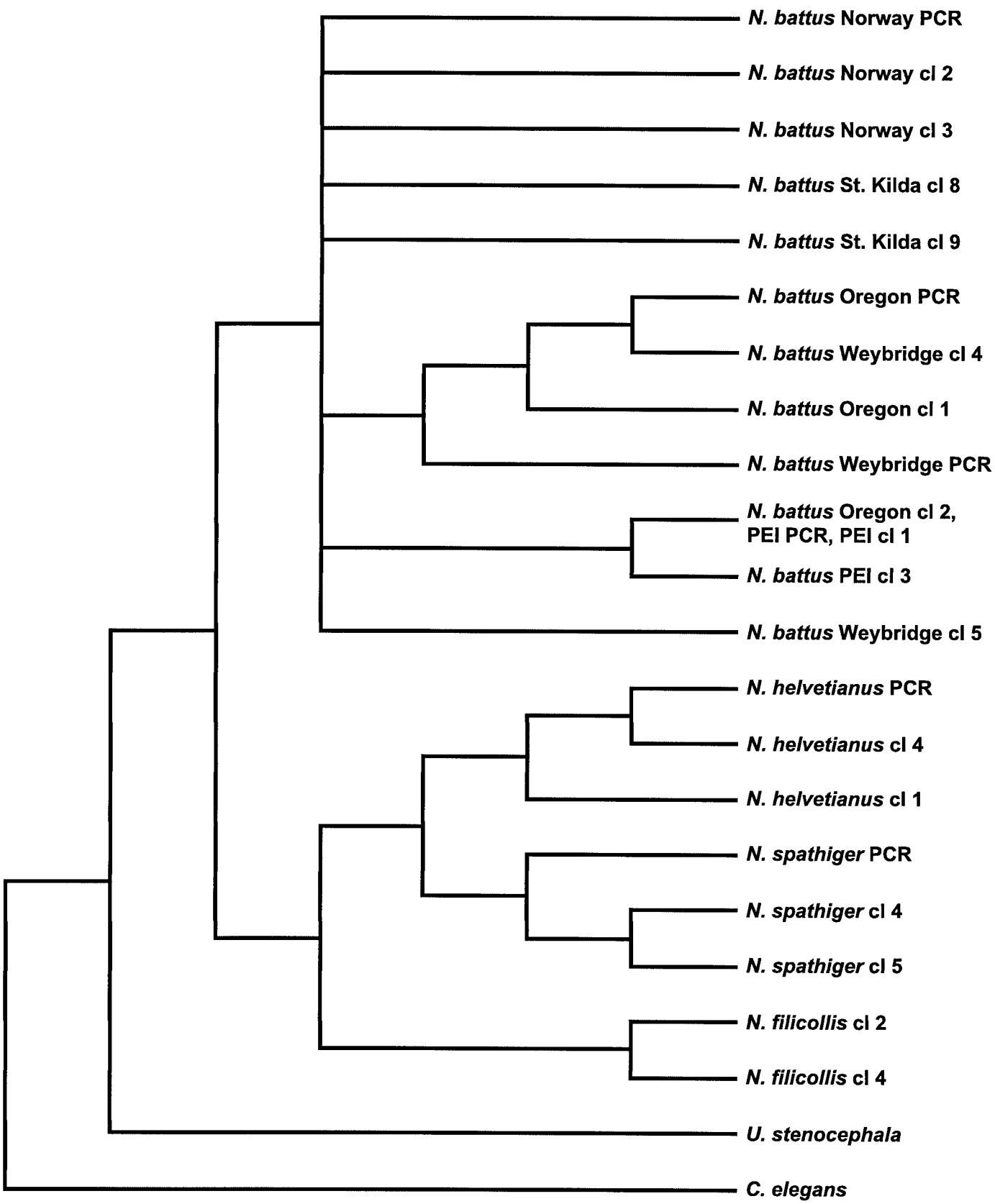

FIGURE 4. Strict consensus of the 2 NJ trees inferred from the standard alignment and the truncated alignment (excluding alignment ambiguous sites), both using log-determinant distances.

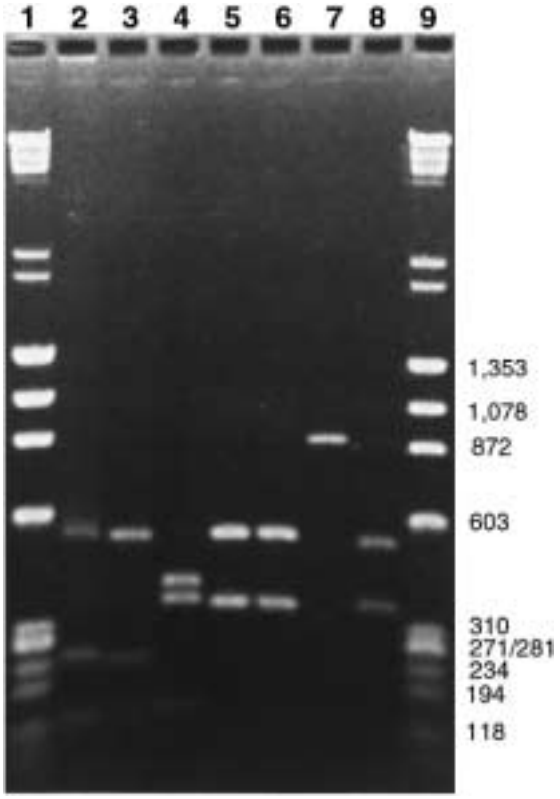

FIGURE 5. Ethidium bromide-stained agarose gel (1.7%) showing diagnostic restriction enzyme patterns for *Nematodirus* species. Lanes 1 and 9 (size markers), lanes 2-6, *Apo* I digests of *N. battus* Norway, *N. battus* Oregon, *N. filicollis*, *N. helvetianus*, and *N. spathiger*; lanes 7 and 8, *BstE* II digests of *N. helvetianus*, and *N. spathiger*.

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

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 relationships among *Nematodirus* species are not directly comparable to results based on biochemical (Rickard et al., 1997) or morphological data (Hoberg, 1997), due to inclusion of different taxa and differences in the types of analyses performed. For example, the preliminary morphological analysis of Hoberg (1997) included 11 *Nematodirus* species, whereas sequence 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 clade consisting of these 2 species and *N. filicollis.* However, the outgroup-rooted trees depicts the *N. battus* sequences as the sister group to ((*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), wherein 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 camelids 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, bovids, and lagomorphs (Hoberg, 1997). The morphological cladogram (Hoberg, 1997) also supports carprine bovids 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 was considerably more widespread in the western Palearctic. The recognition of relictual 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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