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# Molecular phylogenetics and diagnosis of soil and clinical isolates of *Halicephalobus gingivalis* (Nematoda: Cephalobina: Panagrolaimoidea), an opportunistic pathogen of horses

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**Abstract**

Phylogenetic relationships among six isolates of *Halicephalobus gingivalis* (Stefanski, 1954), a species with pathogenic potential in horses and humans, were evaluated using DNA sequences from the nuclear large-subunit ribosomal RNA (LSU rDNA) gene. Sequences from nematodes obtained from in vitro cultures (soil or clinical sources), or isolated from infected horse tissues, were compared. Gene sequences from a fatal equine clinical case from southern California and a free-living isolate recovered from southern California soil showed no fixed differences. Sequences from isolates representing two fatal equine cases from North America, one from Ontario, Canada and another from Tennessee also showed no fixed differences. In contrast, two equine cases from Tennessee had 18 fixed differences for this LSU region, the greatest observed among isolates from horses. Phylogenetic analysis of six *Halicephalobus* sequences and four outgroup taxa by maximum parsimony yielded one tree with five well-supported clades. This phylogeny did not group isolates of *Halicephalobus* strictly by region of geographic isolation or source of sample, and depicted one clinical and one soil isolate as sister taxa. These results confirm that free-living environmental isolates are potential sources of infection for horses. The phylogeny also reveals that diverse isolates can cause infections in horses within a relatively limited geographic region, and conversely that genetically similar sister taxa can be recovered from geographically distant localities. PCR primers that selectively amplify *Halicephalobus* DNA were designed and tested based on comparison of closely related nematodes as inferred from phylogenetic analysis.


*Keywords:* Molecular systematics; *Halicephalobus gingivalis*; PCR diagnostics; Ribosomal DNA

## 1. Introduction

*Halicephalobus gingivalis* (Stefanski, 1954) is a free-living panagrolaimid nematode capable of infecting and reproducing in horses (Stefanski, 1954; Anderson et al., 1998), zebras (Isaza et al., 2000) and humans (Hoogstraten and Young, 1975; Gardiner et al., 1981). The taxonomy of *H. gingivalis* has not been uniform (Anderson et al., 1998), and some relatively recent case reports refer to this species as *Micronema deletrix* or *Halicephalobus deletrix*. However, a detailed redescription of the species and review of the genus has provided convincing evidence for accepting *H. gingivalis* as the valid species name (Anderson et al., 1998). Although *H. gingivalis* appears morphologically distinct among the eight described species of the genus, ratios of body size and measurements of body length fail to clearly distinguish among the other seven nominal species (Anderson et al., 1998), many of which were originally isolated from soil, plants, or moist organic-rich habitats (none as parasites of vertebrates). Due to the small size (235–460 μm) of adults of *Halicephalobus* spp. (Anderson

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et al., 1998) and the apparent absence of distinguishing morphological features among most species (as assessed by light microscopy), DNA-based approaches are needed for delimiting species and studying the phylogenetic relationships among them.

Little has been firmly established concerning the life history or epidemiology of *H. gingivalis*. Reports of infection in horses (and a few in humans) have been published over the last four decades (an equine case listing is provided in Blunden et al., 1987; human cases are described in Gardiner et al., 1981; Table 1 summarises cases reported after 1987); the geographic distribution of these infections suggests that this nematode is cosmopolitan. In horses, parthenogenetic reproduction of nematodes often results in rapid disease progression with clinical signs of infection that may include ataxia and renal dysfunction. Pathological effects in horses commonly include granulomatous inflammation and destruction of infected tissues such as oral or nasal cavities (particularly the mandible, maxilla, or nasal sinuses), spinal cord, eyes, brain, lungs, heart, liver, kidneys, lymph nodes, testes, stomach, and long bones. The death of the host often results from massive proliferation of the nematodes in affected organs (e.g. Blunden et al., 1987; Bröjer et al., 2000; Kinde et al., 2000). There have been only two reports of successful chemotherapeutic treatment of infections of horses (Dunn et al., 1993; Pearce et al., 2001), despite relatively common administration of anthelminthics in cases diagnosed ante mortem.

Table 1
Published cases of *Halicephalobus gingivalis* infections reported since 1987

| Country of origin | Reference |
|---|---|
| United States | Simpson et al., 1988 |
| United States | Darien et al., 1988 |
| Italy | Marocchio and Mutinelli, 1988 |
| Germany | Liebler et al., 1989 |
| Philippines | Alejandro-Matawaran and Peneyra, 1989 |
| Canada | Chalmers et al., 1990 |
| United States | Spalding et al., 1990 |
| United States | Buergelt, 1991 |
| Scotland | Angus et al., 1992 |
| Austria | Reifinger, 1993 |
| United States | Ruggles et al., 1993 |
| United States | Dunn et al., 1993 |
| United States | Trostle et al., 1993 |
| Germany | Schelz, 1993 |
| United States | Rames et al., 1995 |
| United States | Kreuder et al., 1996 |
| Italy | Cantile et al., 1997 |
| Germany | Teifke et al., 1998 |
| Ireland | Weaver et al., 1999 |
| Austria | Wlaschitz et al., 2000 |
| Austria | Majzoub et al., 2000 |
| Ireland | Sturgeon and Bassett, 2000 |
| Germany | Wollanke et al., 2000 |
| Canada | Bröjer et al., 2000 |
| Norway | Aleksandersen et al., 2000 |
| United States | Isaza et al., 2000 |
| United States | Kinde et al., 2000 |
| United States | Wilkins et al., 2001 |
| United States | Johnson et al., 2001 |
| Canada | Pearce et al., 2001 |
| Japan | Shibahara et al., 2002 |
| Italy | Mandrioli et al., 2002 |

Refer to Blunden et al. (1987) for earlier equine cases reports, and Gardiner et al. (1981) for human cases.

The establishment of an in vitro culture of *H. gingivalis* from a recent equine infection (Anderson et al., 1998; Bröjer et al., 2000) has provided a tool for studying the biological and genetic features of a clinical isolate and comparing it with *Halicephalobus* cultures isolated from environmental sources, including soil or compost. In addition, specimens of *H. gingivalis* obtained from other equine infections have yielded DNA suitable for PCR amplification and subsequent sequencing. However, nematodes from other recent clinical cases have not proved useful for establishing additional in vitro cultures. Although the number of *H. gingivalis* isolates available for genetic study are relatively few, the rarity of clinical and soil isolates suitable for molecular study supports using these samples to investigate the genetic relationships among *Halicephalobus* isolates. Such genetic approaches have the potential to aid in identifying basic factors involved in transmission. For example, the hypothesis that isolates of *H. gingivalis* rarely and opportunistically infect hosts would be supported if no genetic distinction exists between representative free-living isolates and those found in infected horses. By contrast, reciprocal monophyly of isolates from horses versus those from soil would be consistent with an infectious pattern of transmission among vertebrates, and would support the proposal that a lineage of *H. gingivalis* from horses may be evolving toward equine host specificity (Blunden et al., 1987).

## 2. Materials and methods

### 2.1. Nematode isolates

Six isolates of *H. gingivalis* were obtained for study (Table 2). Three of these (SAN100, JB128, and JB043) were maintained as in vitro cultures. The SAN100 isolate was originally obtained from a naturally infected horse in Guelph, ON, Canada (Anderson et al., 1998; Bröjer et al., 2000). Isolate JB128 was obtained from a compost pile in Riverside, CA, USA by one of the authors (J.G.B.), and isolate JB043 was recovered from German soil. These three isolates have been continuously subcultured (approximately every 1–3 months, as required) on 1% agar plates without supplements, or 1% agar plates containing 1% Beech-Nut Naturals (mixed cereal stage 2) baby food (Stock et al., 2002).

Specimens of the other three isolates (South Pacific, Tennessee1 and Tennessee2) were recovered from frozen equine tissues representing distinct fatal clinical cases. The

Table 2
*Halicephalobus* spp. isolates used in this study and GenBank accession numbers for large subunit rDNA sequences

| Isolate designation | Origin | Nature of isolate | Accession no. |
|---|---|---|---|
| JB043 | Potting soil containing peat, near Neustadt, Germany, 1995 | Culture | AY294178 |
| JB128 | Vegetable compost pile, Riverside, CA, USA | Culture | AY294181 |
| SAN100 | Equine case, Guelph, Canada; report and description in Anderson et al., 1998 | Culture | AY294177 |
| South Pacific | Equine case, San Bernardino, CA, USA, 1998 | Frozen equine tissues | AY294182 |
| Tennessee1 | Equine case, Tennessee, USA, 2001 | Frozen equine tissues | AY294180 |
| Tennessee2 | Equine case, Tennessee, USA, 2001 | Frozen equine tissues | AY294179 |

South Pacific case (Kinde et al., 2000) was an infection of a Holsteiner stallion from southern California. Specimens of the Tennessee1 isolate were obtained from an infection of a mixed-breed horse (Snider et al., 2001). Nematodes of the Tennessee2 isolate were from an infection of an American miniature mare (C. Patton, personal communication). For these three clinical isolates, preparation of nematodes for morphological examination and genomic DNA extraction were based on specimens collected from frozen horse tissues.

### 2.2. PCR amplification

DNA was extracted from pooled nematodes (10–200 individuals) using commercial kits (IDPure, ID Labs Biotechnology; DNAzol, Molecular Research Center). A region of the 5′-end of the nuclear large subunit ribosomal RNA gene (LSU rDNA) containing the D2 and D3 divergent domains was amplified using forward primer #391 (5′-AGCGGAGGAAAAGAAACTAA) and reverse primer #501 (5′-TCGGAAGGAACCAGCTACTA). PCR reactions (25 μl) consisted of 0.5 μM of each primer, 200 μM deoxynucleoside triphosphates, and $MgCl_2$ ranging from 2 to 3 mM as required to achieve effective amplification. Proof-reading polymerase (0.5 unit, Finnzymes DNAzyme EXT, MJ Research) was used for amplification. PCR cycling parameters included denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, followed by a post-amplification extension at 72 °C for 7 min.

PCR products were prepared for direct sequencing using enzymatic treatment with exonuclease I and shrimp alkaline phosphatase (PCR product pre-sequencing kit, USB Corporation). Sequencing reactions were performed using ABI BigDye v3.0 (PE Applied Biosystems) terminator sequencing chemistry, and reaction products were separated and detected using an ABI 3100 capillary DNA sequencer. PCR products were sequenced using both PCR primers and two internal primers (forward #504, 5′-CAAGTACCGTGAGGGAAAGTTG, and reverse #503, 5′-CCTTGGTCCGTGTTTCAAGACG).

### 2.3. Phylogenetic analyses

Sequences were assembled and base-calling differences resolved using Sequencher version 3.0 (Gene Codes). All sequences were completely double-stranded for accuracy. Site polymorphisms were recorded only when both alternative nucleotide peaks were present in all sequence reactions representing both DNA strands. If the heights of the alternative nucleotide peaks at polymorphic sites were not equal, the height of the minor peak was required to significantly exceed background terminations, and comprise at least 25% of the major peak to be scored as a polymorphism. Sequences representing different isolates and the outgroup species were aligned using CLUSTAL X v1.53b (Thompson et al., 1997) using default pairwise and multiple alignment parameters and the following profile-alignment approach. First, multiple alignment of the *Halicephalobus* isolates was performed using the default alignment parameters. Next, the outgroup species were aligned to the *Halicephalobus* alignment profile, with each outgroup sequence added individually. This procedure produced a multiple alignment of 1,030 characters, which is referred to as the full alignment (FA) dataset. A second data set was produced from the FA by removing 290 sites that were judged (by visual inspection) to be potentially ambiguous with respect to positional homology inference; this is called the without alignment ambiguity (WAA) dataset. Both alignments have been deposited in the TreeBase database (Sanderson et al., 1994), and the individual nucleotide sequences in GenBank (accession numbers in Table 2). Outgroup species were selected based on a comprehensive LSU rDNA molecular phylogeny of cephalob nematodes (Nadler, unpublished results) which strongly supports a monophyletic group of taxa, including *H. gingivalis, Strongyloides* spp., *Rhabditophanes* sp., *Panagrolaimus* sp., and *Turbatrix aceti*, with the clade *Strongyloides* spp. plus *Rhabditophanes* sister to *Halicephalobus*. These molecular phylogenetic results are consistent with the current taxonomic placement of *Halicephalobus* in the Panagrolaimoidea (Andrássy, 1984). Sequences representing these four outgroup genera were included in all analyses: *Rhabditophanes* sp. (KR3021), *Panagrolaimus* sp. (JB115) *Strongyloides*

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                         10         20         30         40         50         60
JB043            AGTAACTGCG AGTGAAAAAG GATAAGCTCA TCGCTGAATC TCTGTGGTTT AAATACTGCA
JB128            .......... ........G. .......... G......... .......... ..T....R..
SAN100           .......... ........G. .......... G......... .......... ..T.......
South Pacific    .......... ........G. .......... G......... .......... ..T.......
Tennessee1       .......... ........G. .......... G......... .......... ..T.......
Tennessee2       .......... .......... .......... G......... .......... ..T.......

                         70         80         90         100        110        120
JB043            GCGAACTGTA GCGTATAAGG ATATATTATG GCATTATATA GATATGTAAG TTCTCGTTGA
JB128            .......... .......G.A G....C.... ...Y...G.. .....C.... ..........
SAN100           .......... .......G.A .....C.... .......G.. .....C.... ..........
South Pacific    .......... .......G.A G....C.... ...C...G.. .....C.... ..........
Tennessee1       .......... .......G.A .....C.... .......G.. .....C.... ..........
Tennessee2       .......... .......G.A .....C.... .......G.. .....C.... ..........

                         130        140        150        160        170        180
JB043            TTCGAGTTCC ATAGAAGGTT TAAGACCTGT AAGATATCTA ATATTTTGTC AATTGATATA
JB128            .......... .A........ .......... ...G...Y.. .C...G.... .....G.T..
SAN100           .......... .A........ .......... ...G...T.. .C...A.... .....G.T..
South Pacific    .......... .A........ .......... ...G...... .C...G.... .....G.T.R
Tennessee1       .......... .A........ .......... ...G...T.. .C...A.... .....G.T..
Tennessee2       .......... .A........ .......... ...G...T.. .C...A.... .....G.T..

                         190        200        210        220        230        240
JB043            CTCTTACGAG TCGGGTTGTT TGGAAACGCA GCCCAAAGTA GGTGGTAAAC TTCATCTAAA
JB128            T......... .......... .......... ........W. .......... .C........
SAN100           T......... .......... .......... .......... .......... .C........
South Pacific    T......... .......... .......... ........W. .......... .C........
Tennessee1       T......... .......... .......... .......... .......... .C........
Tennessee2       T......... .......... .......... .......... .......... .C........

                         250        260        270        280        290        300
JB043            GCTAAATATA ACCGTGAGAC CAATAGTAAA CAAGTACCGT GAGGGAAAGT TGAAAAGTAC
JB128            .......... .......... .......... .......... .......... .......C..
SAN100           .......... .......... .......... .......... .......... .......C..
South Pacific    .......... .......... .......... .......... .......... .......C..
Tennessee1       .......... .......... .......... .......... .......... .......C..
Tennessee2       .......... .......... .......... .......... .......... .......C..

                         310        320        330        340        350        360
JB043            TCTGAAGAGA GAGTTAAAAG AACGTGAAAA CGATTAAGTG GAAGCAAACA GGTCTATTTG
JB128            .......... .......... .......... .......... .......... ........GA
SAN100           .......... .......... .......... .......... .......... ........KR
South Pacific    .......... .......... .......... .......... .......... ........GA
Tennessee1       .......... .......... .......... .......... .......... ........GA
Tennessee2       .......... .......... .......... .......... .......... ..........

                         370        380        390        400        410        420
JB043            AAGGTATGTA TTTATGTATA TATCTGCTAA TATGTATACG AGATGATTAT TAYSTTGTTA
JB128            ...A.G.A.. G..Y.C...G C......... ..CA...GT. GAG.TTC... ..TTC.A..T
SAN100           R....G.R.. G..T.C...R Y......... ..CA...GT. GAG.TTC... ..TTC.A..T
South Pacific    ...A.G.A.. G..C.C...G C......... ..CA...GT. GAGYTWY... ..TTC.A..T
Tennessee1       G....G.... G..T.C...R Y......... ..CA...GT. GAG.TTC... ..TTC.A..T
Tennessee2       ...A...A.. G..T.C.... .......... ..CA...GTA GAGCT..... ..TTC.A..T
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Fig. 1. Alignment of large subunit ribosomal DNA sequences for *Halicephalobus* isolates. Bases that match the first sequence are represented by dots. Positions of primers designed for the diagnostic PCR assay are 68–91 (primer 632), 82–102 (primer 631), 719–740 (primer 634), and 767–789 (primer 633).

*stercoralis* (AL3), and *T. aceti* (Carolina Biological strain). Phylogenetic analyses were performed on both the FA and WAA data sets using methods in PAUP 4.0* v 4.0b 10 (Swofford, 1998), including maximum parsimony with branch-and-bound searches, and neighbour-joining using Hasegawa-Kishino-Yano 85 DNA distances. The pairwise sequence similarity between *Halicephalobus* isolates was assessed by the absolute number of nucleotide differences (non-conflicting polymorphisms, e.g. G versus A/G, counting as a match). Relative reliability of clades in parsimony and neighbour-joining trees was assessed by bootstrap resampling (2,000 replicates).

### 2.4. Diagnostic PCR

Sequences of two forward (#631, 5′-TATACTATGGCATTATGTAGA; #632, 5′-GTAGCGTATAGGAATATACTATGG) and two reverse (#633, 5′-CGATTTGCACG-

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                    430        440        450        460        470        480
JB043               TAAGAGGACT CCAATTATAT TGAAGAAACC ATATAATGAT TGAACATTAT TCTCTTATAC
JB128               .......... .---.---.. ATG....C.. T...G...G. .....G..R. CTC.C....T
SAN100              .......... .AC..---.. GTG....C.. T.......G. .....G.... CTC.C....T
South Pacific       .......... .---.---.. ATG....C.. T...G...G. .....G..R. CTC.C....T
Tennessee1          .......... .AC..---.. GTG....C.. T.......G. .....G.... CTC.C....T
Tennessee2          .......... .---.---.. ATG....C.. T...R...G. .....G..G. CTCTG....T

                    490        500        510        520        530        540
JB043               GATTATTAGC ATTTATGATR GTTACCTGTT TGACCCGTCT TGAAACACGG ACCAAGAAGT
JB128               ..A....... .........A .......... .......... .......... ..........
SAN100              ..A....... .........A .......... .......... .......... ..........
South Pacific       ..A....... .......... .......... .......... .......... ..........
Tennessee1          ..A....... .........A .......... .......... .......... ..........
Tennessee2          ..A....... .........G .......... .......... .......... ..........

                    550        560        570        580        590        600
JB043               TTATTGTATA CGCGAGTCAT TAAGTGTAAA AACTTATTGG CGTAATGAAA GTGAAGGTTT
JB128               .......... .......... .......T.. .......... .A........ ..A......C
SAN100              .......... .......... .......T.. .......... .A........ ..A......C
South Pacific       .......... .......... .......T.. .......... .A........ ..A......C
Tennessee1          .......... ...R...... .......T.. .......... .A........ ..A......C
Tennessee2          .......... .......... .......... .......... .A........ ..A......Y

                    610        620        630        640        650        660
JB043               ATTTGTTAAG CTTACGTATG ATAATGTATT TACGGATATA TTAGTAATAC GGTCCTATTC
JB128               ...C...G.. ..G.T..... ....A..G.. ......C..T ....C.G... A.........
SAN100              .C.....G.. ..A.T..... ....A..G.. ......C..T ....C.G... A.........
South Pacific       .Y.C...G.. ..G.T..... ....A..G.. ......C..T ....C.G... A.........
Tennessee1          .C.....G.. ..R.T..... ....A..G.. ......C..T ....C.G... A.........
Tennessee2          .C.....R.R ..G.T..... ....A..G.. ......C..T ....C.G... A.........

                    670        680        690        700        710        720
JB043               TAATGATTTA TCATAGAGTG GAGATAGAGC GTATGCAATG AGACCCGAAA GATGGTGATC
JB128               .T...GC..G C...G..... .......... .......... .......... ..........
SAN100              .T...GC..G C...G..... .......... .......... .......... ..........
South Pacific       .T...GC..G C...G..... .......... .......... .......... ..........
Tennessee1          .T...GC..G C...G..... .......... .......... .......... ..........
Tennessee2          .T...GC..G C...G..... .......... .......... .......... ..........

                    730        740        750        760        770        780
JB043               TATGCTTGAG CAGGATGAAG CCGGAGGAAA CTCTGGTGGA AGTCCGTAAC GGTTCTGACG
JB128               .......... .......... .......... ........A. .......... ..........
SAN100              .......... .......... .......... ........A. .......... ..........
South Pacific       .......... .......... .......... ........A. .......... ..........
Tennessee1          .......... .......... .......... ........A. .......... ..........
Tennessee2          .......... .......... .......... .......... .......... ..........

                    790        800        810        820
JB043               TGCAAATCGA TCGTCTGACT TGAGTATAGG GGCGAAAGAC
JB128               .......... .......... .......... ..........
SAN100              .......... .......... .......... ..........
South Pacific       .......... .......... .......... ..........
Tennessee1          .......... .......... .......... ..........
Tennessee2          .......... .......... .......... ..........
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Fig. 1 (*continued*)

TCAGAACCGTTA; #634, 5′-CTTCATCCTGCTCAAGCATAGA) oligonucleotide primers, designed to selectively amplify *Halicephalobus* DNA, were chosen based on a multiple alignment of LSU sequences from 12 additional genera from the suborder Cephalobina (*Brevibucca, Cervidellus, Cephalobus, Macrolaimellus, Panagrellus, Panagrolaimus, Pseudacrobeles, Rhabditophanes, Steinernema, Strongyloides, Turbatrix* and *Zeldia*). These primers were designed to reduce the likelihood of non-target sequence PCR amplification based on 3′-end primer:template mismatch amplification efficiencies (Kwok et al., 1990). Primer combinations were screened initially for *Halicephalobus* specificity using DNA from *Turbatrix aceti* and *Plectonchus hunti*. Subsequently, 10 cephalob genera (*Acrobeles complexus, Cephalobus persegnis, Panagrellus redivivus, Panagrolaimus* sp., *Plectonchus hunti, Stegelleta* sp., *Steinernema feltiae, Strongyloides stercoralis, Turbatrix aceti* and *Zeldia punctata*), and two DNA extracts prepared from mixtures of nematodes obtained from culture were used to test the specificity and sensitivity of PCR. One

Table 3
Pairwise nucleotide distances (absolute differences) and number of rDNA sequence polymorphisms for *Halicephalobus* isolates

| | Isolate | Polymorphisms | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|---|
| 1 | SAN100 | 6 | – | | | | | |
| 2 | JB043 | 3 | 73 | – | | | | |
| 3 | Tennessee2 | 4 | 15 | 69 | – | | | |
| 4 | Tennessee1 | 4 | 0 | 76 | 18 | – | | |
| 5 | JB128 | 6 | 8 | 78 | 19 | 9 | – | |
| 6 | South Pacific | 8 | 10 | 77 | 17 | 11 | 0 | – |

mixed nematode DNA extract contained 50% *H. gingivalis* individuals (five *H. gingivalis* JB128, three *Cephalobus* persegnis BSS5 and two *Panagrolaimus* sp. JB034), whereas the second mixture was 10% *H. gingivalis* (one *H. gingivalis* JB128, five *Cephalobus persegnis* BSS5 and four *Panagrolaimus* sp. JB034). DNA extracts of the cephalob genera used to test PCR specificity were also screened with a second set of LSU rDNA PCR primers (#537 forward, 5′-GATCCGTAACTTCGGGAAAAG-GAT; #531 reverse, 5′-CTTCGCAATGATAGGAA-GAGCC) as positive controls to ensure that these extracts were suitable for amplification. PCR reaction mixtures and amplification conditions were as described for primers 391/501, except that a concentration of 3 mM $MgCl_2$ were used throughout.

## 3. Results

### 3.1. Sequence data, pairwise differences and apomorphy analysis

All of the LSU sequences (GenBank AY294177–86), including the outgroup species, were characterised by high A + T content (Fig. 1). For the *Halicephalobus* isolates, A + T ranged from 60% (South Pacific) to 63.8% (JB043). Similarly, the A + T% of *S. stercoralis* and *Rhabditophanes* were 71.7% and 58.7%, respectively. The *Halicephalobus* isolates were characterised by 3–8 sequence polymorphisms (Fig. 1 and Table 3); outgroup species had 0–3 sequence polymorphisms. Because pooled nematodes were the source of DNA for PCR reactions, it cannot be determined if the observed sequence polymorphisms represent variation among individual nematodes, or intra-individual variation. The absolute number of nucleotide differences between *Halicephalobus* isolates (Table 3) ranged from 0 to 78 (0–19 among the North American isolates). No fixed sequence differences were found between the South Pacific and JB128 isolates, or the Tennessee1 and SAN100 isolates. Based on the maximum parsimony tree topology, unambiguous nucleotide autapomorphies for *Halicephalobus* isolates ranged from zero to 28 (Fig. 2).

### 3.2. Phylogenetic analyses

Branch-and-bound parsimony analysis of the LSU rDNA FA data set yielded a single most parsimonious tree (Fig. 2). This data set included 289 parsimony-informative characters, required 853 steps and had a consistency index of 77% (excluding uninformative characters). The bootstrap majority-rule consensus tree for the FA data set was identical in topology to the maximum parsimony tree (Fig. 2) and had relative support ranging from moderately high to very high for ingroup clades. For example, monophyly of the North American *Halicephalobus* isolates was recovered in 100% of the bootstrap replicates, and the California horse (South Pacific) and soil (JB128) isolates were sister taxa in 98% of the trees. The maximum parsimony tree does not represent the two Tennessee horse isolates as a monophyletic group, and this result is strongly supported by bootstrap resampling. A clade of four taxa in the maximum parsimony tree (South Pacific, JB128, SN100, and Tennessee1) was further subdivided into two sister clades, one containing South Pacific and JB128, and the other containing SAN100 and Tennessee1, with the latter clade receiving moderately high bootstrap support (88%). The neighbour-joining tree for the FA dataset (Fig. 3) had the same topology as the maximum parsimony tree. Bootstrap percentages of clades in the neighbour-joining tree were equal or higher in comparison to the maximum parsimony bootstrap tree (Fig. 3). With the exception of *H. gingivalis* JB043 and the outgroup species, inferred branch lengths in the neighbour-joining tree were short.

When alignment-ambiguous characters were excluded from the analyses (WAA dataset, 241 parsimony-informative characters), branch-and-bound parsimony analysis recovered a single tree of 575 steps with a consistency index of 76%. The topology of this tree was the same as in Fig. 2 for the ingroup; relationships among the outgroup species were slightly different ((*Panagrolaimus* sp., *T. aceti*), (*Rhabditophanes* sp., *S. stercoralis*)). The bootstrap majority-rule consensus tree for the WAA dataset was identical in topology to the WAA maximum parsimony tree. Bootstrap percentages for ingroup clades in the WAA analysis (Fig. 2) were typically only slightly lower than found for the FA analysis. The neighbour-joining tree for the WAA dataset had the same topology as the FA neighbour-joining tree; bootstrap percentages for clades in the WAA neighbour-joining tree were lower for two nodes (Fig. 3).

### 3.3. Halicephalobus gingivalis-specific PCR

All four PCR primer-pair combinations designed to be *Halicephalobus*-specific yielded products of their predicted sizes with *H. gingivalis* DNA. One of these primer combinations (632/633) also amplified a PCR product from *Turbatrix* DNA in initial specificity assessments. Subsequent development and testing of the specific PCR

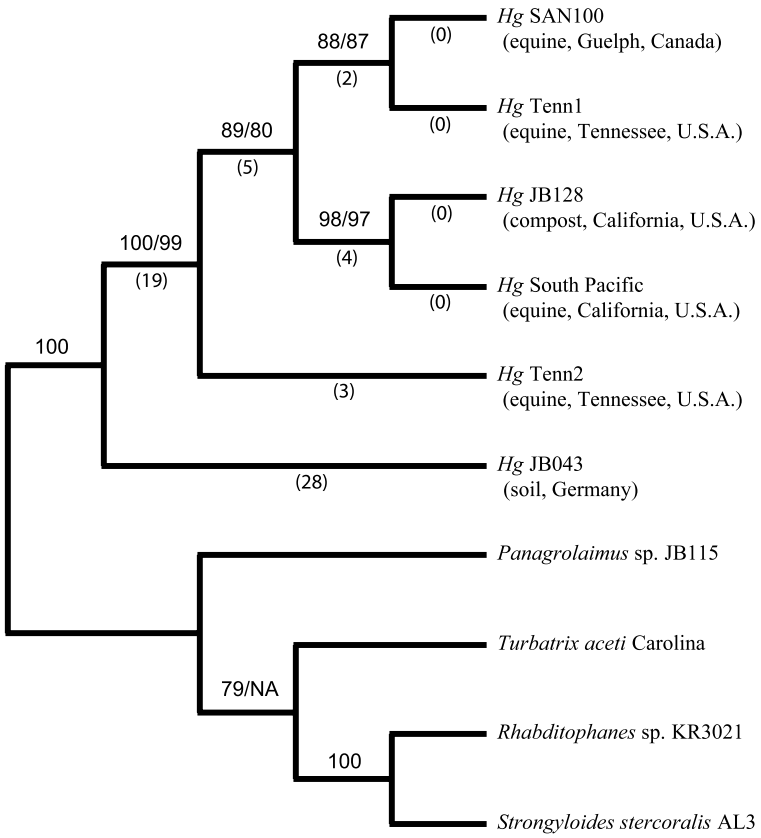


Fig. 2. Maximum parsimony tree depicting phylogenetic relationships among six *Halicephalobus* isolates and outgroups based on full alignment (FA) dataset (tree length 853 steps, 289 parsimony informative characters, consistency index = 77%). The most parsimonious tree for the without alignment ambiguity (WAA) dataset had the same topology for the *Halicephalobus* taxa. Bootstrap percentages of clades as inferred by maximum parsimony inference are shown above internal nodes; values for the FA dataset are listed first, with values for the WAA dataset following the slash. The number of unambiguous apomorphies in the FA dataset for internal and external nodes of *Halicephalobus* is given below nodes in parentheses. *Hg, H. gingivalis.*

used the 632/634 primers (673 bp amplicon) because they yielded the most abundant product in *H. gingivalis* control amplifications. The nucleotide sequence of this amplicon (GenBank AY295803) corresponded to the LSU region of *H. gingivalis* that served as the template for primer design. The 632/634 primers also yielded strong product from both mixed nematode DNA extracts, including the mixture containing one (10%) *H. gingivalis* (Fig. 4). PCR amplifications with the 632/634 primers failed to yield any detectable product (regardless of size) using DNA from *Acrobeles complexus, Cephalobus persegnis, Panagrellus redivivus, Panagrolaimus* sp., *Plectonchus hunti, Stegelleta* sp., *Steinernema feltiae, Strongyloides stercoralis, Turbatrix aceti* and *Zeldia punctata*. These DNA samples had previously been successfully used for rDNA amplification using other LSU primers (Fig. 4).

## 4. Discussion

Based on morphological and morphometric criteria, *H. gingivalis* is distinct among the eight described species of *Halicephalobus*, whereas the other seven species are very similar to each other, with few if any convincing morphological or morphometric differences (Anderson et al., 1998). Although *H. gingivalis* has most typically been isolated and reported from tissues of infected horses (the source of its original description), the other described species of *Halicephalobus* have never been isolated from vertebrates, and instead were described following their isolation from soils, rotting wood, plants, fresh or saltwater environments (Andrássy, 1984; Geraert et al., 1988). Given the apparent paucity of morphological differences among most nominal species of *Halicephalobus*, delimiting and distinguishing valid species requires genetic data.

Phylogenetic analysis of LSU rDNA sequences revealed that isolates representing the morphospecies *H. gingivalis* consist of more than one genetically distinct evolutionary lineage, although *Halicephalobus* isolates share a most recent common ancestor based on the rDNA phylogeny. These results are supported by analyses employing conservative criteria for inferring positional homology of rDNA sequences. Cladistic analysis of LSU characters revealed strong support for four evolutionary lineages (potential species) of *Halicephalobus* that were delimited based on apomorphies (Fig. 2), including: (1) SAN100 plus

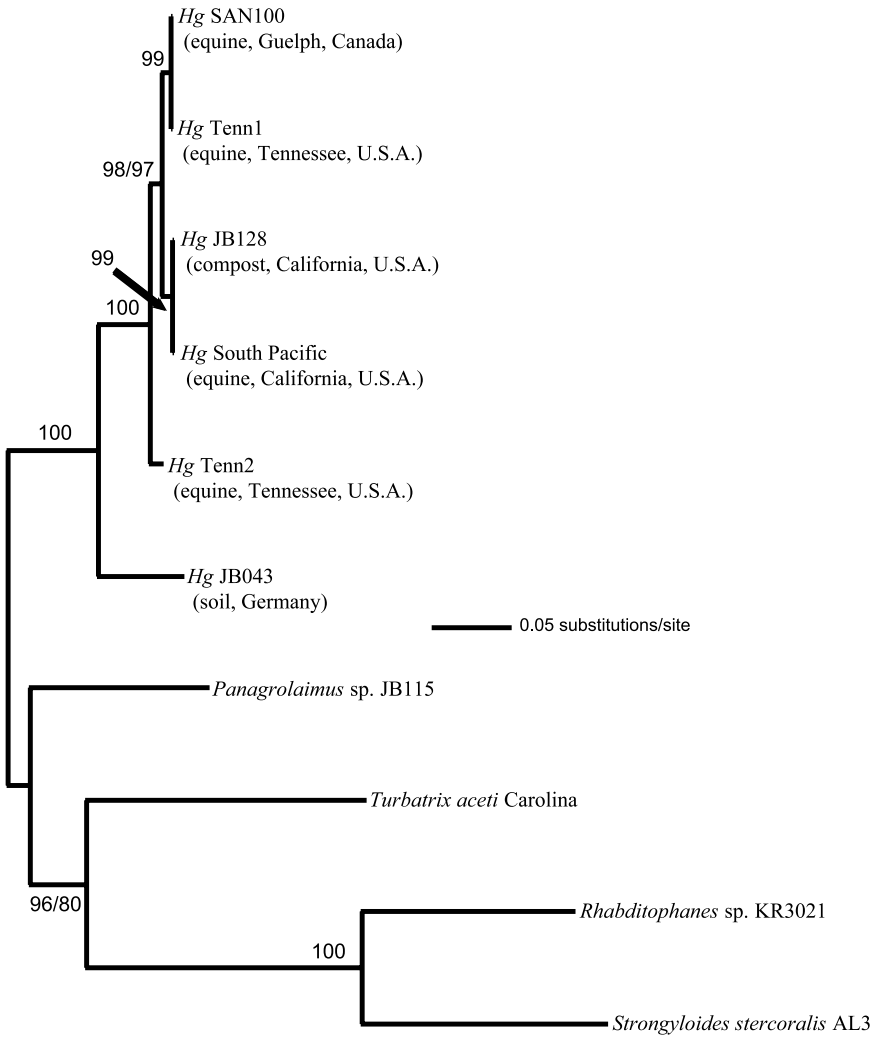


Fig. 3. Neighbour-joining distance tree for *Halicephalobus gingivalis* (*Hg*) isolates and outgroups based on Hasegawa-Kishino-Yano 85 distances and the full alignment (FA) dataset. The neighbour-joining tree for the without alignment ambiguity (WAA) dataset had the same topology. Branch lengths are scaled to represent substitutions per site. Bootstrap percentages of groups are shown near nodes, with values for the FA dataset listed first, followed by values for the WAA dataset, when different.

Tennessee1, (2) JB128 plus South Pacific, (3) Tennessee2, and (4) JB043. However, delimiting species based only on interpretation of these rDNA data would not be prudent because testing hypotheses of species using gene trees is best accomplished by analysis of multiple loci and corroboration of lineage independence from concordant patterns (Nadler, 2002). In addition, a sufficient number of individual organisms must be sequenced to establish that inferred apomorphic characters represent fixed character states rather than potential polymorphisms (Adams, 1998; Nadler et al., 2000a, 2002). In this study, pooled nematodes were the source of DNA used for PCR, and therefore, it is not possible to establish, with confidence, whether particular nucleotide character states are fixed, since it is unknown how many individuals are represented by the sequence obtained from the PCR product. Likewise, although the number of observed rDNA polymorphisms observed in *Halicephalobus* isolates seems high, it cannot be determined whether these polymorphisms represent intra-individual rDNA repeat variation (Nadler et al., 2000b), or variation among individuals in the pooled sample. Indeed, because inferences concerning lineage independence of *Halicephalobus* isolates have been based on pooled individuals and data from one locus, it is premature to interpret this tree and character analysis as a definitive test of the number of species represented by these isolates. Nevertheless, this rDNA gene tree provides evidence for the existence of distinct genetic lineages, which suggests that a multilocus study of individuals representing these and other isolates is warranted to fully test the hypothesis of cryptic species within the morphotype of *H. gingivalis*. It would also seem warranted to study the morphology of these distinct *Halicephalobus* lineages in greater detail, although preliminary scanning electron microscopic observations on

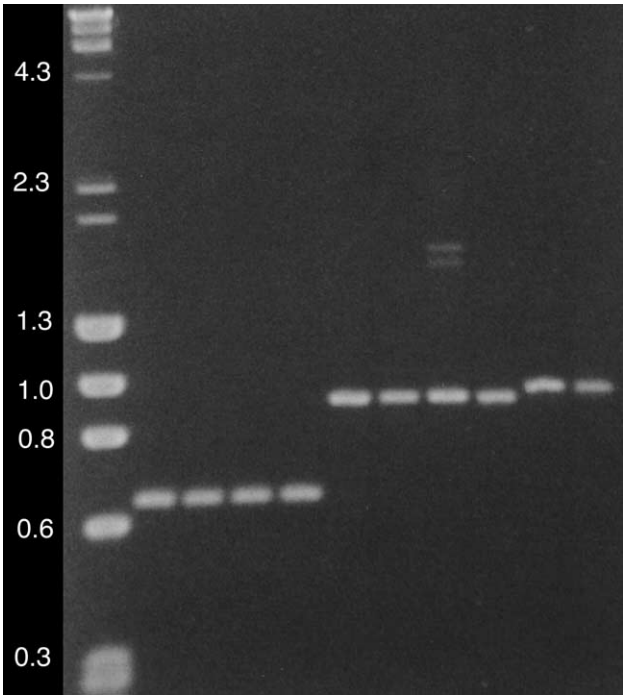


Fig. 4. Ethidium bromide-stained agarose gel (1.4%) showing diagnostic PCR for *Halicephalobus gingivalis* (673 bp product) and control reactions demonstrating the sensitivity and specificity of primer pair 632/634. Note that lanes 6–11, which are tests of primer specificity, are double-loaded with reaction products from 632/634 PCRs (all negative) and large subunit ribosomal DNA positive control PCRs (primers 537/531, yielding products of ~960 bp). Lane 1, size markers indicated in kb; lane 2, *H. gingivalis*, DNA = bulk worm extract; lane 3, *H. gingivalis*, DNA = extract of 10 nematodes; lane 4, DNA = extract of five *H. gingivalis*, three *Cephalobus persegnis* BSS5 and two *Panagrolaimus* sp. JB034; lane 5, DNA = extract of one *H. gingivalis*, five *Cephalobus persegnis* BSS5 and four *Panagrolaimus* sp. JB034; lanes 6–11, double-loaded with 632/634 diagnostic PCR and LSU rDNA control reaction of: lane 6 (*Cephalobus persegnis* BSS5), lane 7 (*Panagrolaimus* sp. JB034), lane 8 (*Plectonchus hunti* JB033), lane 9 (*Turbatrix aceti*), lane 10 (*Panagrellus redivivus* JB052), lane 11 (*Strongyloides stercoralis*).

nematodes from isolates JB043, JB128, and SAN100 showed no apparent differences in external anatomy (unpublished data).

Phylogenetic analysis of LSU sequences revealed that certain groups of isolates represent single lineages, and pairwise distances document that some of these isolates lack fixed genetic differences for this region of rDNA. For example, rDNA gene sequences from the South Pacific isolate, representing a fatal equine case from southern California (Kinde et al., 2000), and sequences from a free-living isolate recovered from a southern California compost pile (JB128) showed no fixed differences and, as might be expected, were strongly supported as sister taxa in phylogenies. This genetic finding is consistent with the expectation that free-living environmental isolates are sources of infection for horses. In addition, sequences from nematodes representing two other fatal equine cases from North America, one from Ontario, Canada (SAN100) and another from Tennessee USA (Tennessee1) also showed no fixed differences and were sister taxa in phylogenetic trees. This result indicates that isolates of *H. gingivalis* that would appear to be genetically homogeneous (based on rDNA) are not narrowly restricted in geographical distribution. Conversely, two equine cases from Tennessee had 18 fixed differences for this LSU region (the greatest number observed among isolates from horses), and were not the closest relatives of one another in the phylogenetic tree. This result shows that single geographic regions may contain a diverse pool of isolates capable of infecting horses, and that halicephalobiasis is caused by a genetically diverse group of infectious agents. However, the full geographic range for any genetic lineage of *H. gingivalis* is unknown. Notably, the phylogenetic analysis does not reveal distinct clades corresponding to free-living and parasitic isolates, a result that would have been expected if there had been an exclusive pattern of infectious transmission among horses for certain lineages with concomitant evolution toward equine host specificity (Blunden et al., 1987).

To date, identification of *Halicephalobus* in soil or other environmental samples (other than infected horses) has required labour-intensive microscopic identification of all small adult panagrolaimid nematodes present in the material. Screening of nematode samples for *Halicephalobus* may be facilitated using the set of specific PCR primers (numbers 632 and 634) designed based on comparison of *Halicephalobus* sequences to a set of sequences that included the four outgroup species and eight additional species of free-living soil microbivores. These taxa were selected for comparison to *H. gingivalis* based on their phylogenetic affinities, and thus it is reasonable to expect that these closely related nematodes would be most informative for guiding the design of primers for taxon-specific (e.g. *Halicephalobus*) amplification. This PCR identification method should prove useful in providing definitive genetic diagnosis of halicephalobiasis for presumptive case histories when biopsy material is available. Although these primers did not amplify DNA from 10 nematode genera (several of which are commonly recovered from soils), including genera most closely related to *Halicephalobus* as determined from phylogenetic analysis, this testing did not include representatives of certain other clades that are common soil inhabitants (e.g. Rhabditinae). Thus, the specificity, sensitivity and practicality of this PCR method of detecting *Halicephalobus* in environmental samples (that will contain PCR inhibitors) must be confirmed through additional field-testing, with confirmation of positive products by both sequencing and recovery of nematodes that are consistent with the morphology of *Halicephalobus*. Such a PCR screening process has the potential to rapidly identify the presence of *Halicephalobus* in samples enriched for nematodes (e.g. using a Baermann funnel; Baermann, 1917), which would facilitate the collection of data on the distribution of this

organism. Such information would be valuable for understanding the potential for transmission to susceptible vertebrates, particularly considering the rarity, yet widespread geographic distribution of halicephalobiasis in horses.

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