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Phylogenetic Relationships Among *Xiphinema* and *Xiphidorus* Nematode Species from Brazil Inferred from 18S rDNA Sequences

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Abstract: Maximum likelihood trees produced from 18S rDNA sequences separated 14 *Xiphinema* and five *Xiphidorus* nematode species from Brazil into distinct groups that concurred with their current morphological taxonomic status. Species belonging to the *X. americanum* group (*X. brevicolle, X. diffusum, X. oxycaudatum,* and *X. peruvianum*) formed a single group that was clearly separated from the other *Xiphinema* species. As with previous taxonomic studies that noted only minor morphological differences between putative *X. americanum* group species, separation of these species based upon 18S rDNA sequences was inconclusive. Thus it is probable that instead of comprising distinct species, the *X. americanum* group may in fact represent numerous morphotypes with large inter- and intra- population morphological variability that may be environmentally driven. Within the cluster representing non *X. americanum* group species, there was little statistical support to clearly separate species. However, three subgroups, comprising (i) the *X. setariae/vulgare* complex, (ii) *X. ifacolum* and *X. paritaliae,* and (iii) *X. brasiliense* and *X. ensiculiferum* were well resolved.

Key words: 18S rDNA, longidorid, molecular systematics, plant-parasitic nematode, ribosomal DNA, *Xiphinema, Xiphidorus.*

Ectoparasitic Longidoridae are globally an economically important family of nematodes that cause damage to an extensive range of crop plants by their feeding on plant root cells. However, more important are those longidorid species that transmit viruses to a wide range of fruit and vegetable crops (Brown et al., 1995, 1996; Ferraz and Brown, 2002; Taylor and Brown, 1997; Weischer and Brown, 2000).

Given that only 18 longidorid species are virus vectors (Brown and Weischer, 1998), there has been a particular necessity to accurately identify longidorid species as remedial agronomic practices differ depending on the presence of virus-vector rather than non-vector longidorid species. Thus, during the last 30 to 40 years, longidorids have arguably received greater taxonomic study than any other nematode group (Taylor and Brown, 1997).

This level of taxonomic study has led to a concomitant increase in the number of both *Longidorus* and *Xiphinema* species, many of which are similar morphologically and morphometrically (Taylor and Brown, 1997; Coomans et al., 2001). In particular, taxonomic controversy surrounds the *Xiphinema americanum* group (Brown and Halbrendt, 1997; Lamberti et al., 2000; Luc and Baujard, 2001). A number of recent studies have tried to elucidate taxonomic relationships by using morphometric data in both statistical (Lamberti et al., 2002) and classical phylogenetic (Coomans et al., 2001) analyses. However, few such studies have used molecular data.

18S rDNA, also known as small subunit (SSU) rDNA, is frequently used for both phylogenetic analyses and diagnostic purposes because it has both conserved and variable regions (Dorris et al., 1999). This region is approximately 1700 base pairs (bp) in length with approximately 50% of the nucleotide variability of the whole gene toward the 5′ end, as it encompasses both conserved stem and highly divergent loop regions. This pattern of conservation and divergence recommends it for analysis, as the region is of a relatively constant length and can be aligned with confidence (Floyd et al., 2002). The conserved regions allow sequences from divergent taxa to be easily aligned and also enable the design of universal primers. The variable regions can be used to distinguish taxa (Dorris et al., 1999). In addition, data from a considerable number of species of nematodes are available for comparative studies (Blaxter et al., 1998; Schierenberg, 2000).

Molecular phylogenies of free-living, plant- and animal-parasitic nematodes have been produced by several authors based on 18S rDNA (Aleshin et al., 1998; Blaxter et al., 1998; De Ley et al., 2002; Kampfer et al., 1998; Kanzaki and Futai, 2002). In one study, 18S rDNA sequences from nematode species representing all major families were compared and a taxonomic re-evaluation within the Phylum Nematoda was suggested (Blaxter et al., 1998). Also, based on 18S rDNA sequences, Hübschen et al. (2002) investigated the phylogeny of predominantly European Longidoridae. Moreover, the evolutionary relationships among members of Dorylaimida were studied using approximately 600bp of the total 18S rDNA (Mullin et al., 2002).

Longidoridae is comprised of six genera: *Longidoroides* Khan, Chawla and Saha, 1978; *Longidorus* Micoletzky, 1922; *Paralongidorus* Siddiqi, Hooper and Khan, 1963; *Paraxiphidorus* Coomans and Chaves, 1995; *Xiphi-*

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dorus, Monteiro, 1976; and *Xiphinema* Cobb, 1913 (Coomans, 1996; Doucet et al., 1998).

Currently, all longidorid genera, except *Paralongidorus,* have been reported from Latin America (Doucet et al., 1998). Of those five genera, only two, *Xiphidorus* and *Xiphinema,* had species recorded during a survey of disparate habitats in Brazil (Oliveira et al., 2003); here, we report their phylogenetic relationships based on 18S rDNA sequences.

Materials and Methods

Nematodes: Soil samples were collected from a range of habitats (Table 1) from all five regions of Brazil as described by Oliveira et al. (2003). Longidorid nematodes (*Xiphinema* and *Xiphidorus* species) were collected from all sampled regions. Nematodes were extracted from soil samples by a modified decanting and sieving technique (Ploeg and Brown, 1997), and those specimens to be used for molecular studies were handpicked and placed live directly into 1M NaCl. These samples were kept at −20 °C and transported to Dundee, Scotland. Specimens used for morphological study were prepared as permanent mounts (Hooper, 1986), identified using light microscopy, and retained as a reference data bank for the molecular studies.

DNA Extraction: DNA from a minimum of two individual adult females of each of 14 *Xiphinema* species and five *Xiphidorus* species from Brazil (Table 1) was extracted using a modified method described by Stanton et al. (1998).

Individual nematodes were placed into separate 0.5-ml micro-centrifuge tubes each containing 20 µl 0.25M NaOH and incubated at 25 °C overnight. Thereafter, samples were incubated at 99 °C for 3 minutes, and 10 µl 0.25M HCl, 5 µl 0.5M Tris-HCl, (pH 8.0), and 5µl 2% Triton X-100 were added to each tube. Samples were incubated at 99 °C for a further 3 minutes. Also, DNA from *X. americanum sensu stricto* from South Africa and *Mononchus aquaticus* Coetzee 1968 from India was extracted and included in the present study to determine intra-group variability and to act as an outgroup, respectively.

18S rDNA PCR amplification: Two Ready-to-Go PCR beads (Amersham International, Little Chalfont, UK) were placed into a 0.2-ml micro-centrifuge tube and 47.5 µl distilled water; 0.5 µl template DNA and 1 µl of each 10 µM primer pair were added. The full-length 18S rDNA was amplified in three fragments using combinations of different primers (Table 2). PCR conditions were as follows: 94 °C for 2 minutes 45 seconds, then 40 cycles of 94 °C for 1 minute, 57 °C for 45 seconds, and 72 °C for 2 minutes. The final extension phase was 72 °C for 10 minutes. PCR products were separated on 1% agarose gel and visualized by staining with ethidium bromide. If PCR products were of sufficiently high quality, they were purified for sequencing using the protocol listed by the manufacturer (Qiaquick PCR Purification Kit, QIAGEN Inc., Crawley, West Sussex, UK).

Sequencing: Purified DNA fragments were sequenced directly in both directions using each primer pair, one forward and one reverse, using a Big Dye Terminator cycle sequencing kit (Applied Biosystems, Warrington, UK), according to the manufacturer's instructions. For each sequencing reaction the following reagents were added to a 0.5-ml micro-centrifuge tube: 4 µl terminator Ready Reaction Mix, 1 µl primer (3.4 µM), and 5 µl

Table 1. *Xiphinema* and *Xiphidorus* species from Brazil (Oliveira et al., 2003) included in this study.

Species	Host	Locality (City, State)	Accession number (GenBank)
Xiphinema brevicolle Lordello and Costa, 1961	*Coffea arabica*	São Paulo, SP	AY297822
Xiphinema diffusum Lamberti and Bleve-Zacheo, 1979	*Prunus persica*	Pelotas, RS	AY297823
Xiphinema oxycaudatum Lamberti and Bleve-Zacheo, 1979	Natural vegetation	Pelotas, RS	AY297835
Xiphinema peruvianum Lamberti and Bleve-Zacheo, 1979	*Coffea arabica*	Dourados (Indápolis), MS	AY297832
Xiphinema brasiliense Lordello, 1951	*Euterpes edulis*	Cananéia, SP	AY297836
Xiphinema elongatum Schuurmans Stekhoven and Teunissen, 1938	*Saccharum officinarum*	Arez, RN	AY297824
Xiphinema ensiculiferum (Cobb, 1893) Thorne, 1937	Natural vegetation	Guarantã, MT	AY297825
Xiphinema krugi Lordello, 1955 (Tail digitate)	Natural vegetation	Dourados, MS	AY297828
Xiphinema krugi Lordello, 1955 (Tail subdigitate)	*Eugenia uniflora*	Florianópolis, SC	AY297827
Xiphinema longicaudatum Luc, 1961	*Brachiaria decumbens*	Amapáh, AP	AY297829
Xiphinema ifacolum Luc, 1961	Natural vegetation	Castanhal, PA	AY297826
Xiphinema paritaliae Loof and Sharma, 1979	*Psidium guajava*	Una, BA	AY297831
Xiphinema surinamense Loof and Maas, 1972	*Carapa guianensis*	Guarapuava, PR	AY297833
Xiphinema setariae/vulgare complex	*Citrus* sp.	Piracicaba, SP	AY297840
Xiphinema variegatum Siddiqi, 2000	*Manihot esculenta* and *Zea mays*	Laranjal do Jari, AP	AY297834
Xiphidorus balcarceanus Chaves and Coomans, 1984	Cerrado	Guia Lopes da Laguna, MS	AY297839
Xiphidorus minor Rashid, Coomans and Sharma, 1986	Natural vegetation	Piracicaba, SP	AY297830
Xiphidorus yepesara parthenus Monteiro, Lordello and Nakasono, 1981	*Saccharum officinarum*	São Pedro, SP	AY297837
Xiphidorus yepesara yepesara Monteiro, 1976	Natural vegetation	Bonito, MS	AY297838
Xiphidorus sp.	Natural vegetation	Dourados, MS	AY297841

Table 2. Oligonucleotide primers used to elucidate 18S rDNA sequences. Primer source is denoted by superscript.

Oligo name	Primer sequence (5′ – 3′)	Direction
SSU_F_04[a]	GCTTGTCTCAAAGATTAAGCC	Forward
SSU_F_07[a]	AAAGATTAAGCCATGCATG	Forward
SSU_R_09[a]	AGCTGGAATTACCGCGGCTG	Reverse
SSU_F_22[a]	TCCAAGGAAGGCAGCAGGC	Forward
SSU_R_13[a]	GGGCATCACAGACCTGTTA	Reverse
SSU_F_02[a]	GGAAGGGCACCACCAGGAGTGG	Forward
SSU_R_81[a]	TGATCCWKCYGCAGGTTCAC	Reverse
XIPHF[b]	CGGTCCAAGAATTTCACCTC	Reverse
XIPHR[b]	GGAGAGGGAGCCTGAGAAAC	Forward

[a] http://nema.cap.ed.ac.uk/biodiversity/sourhope/nemoprimers.html
[b] Designed for this study using PRIMER3 software, (http://wwwgenome.wi.mit.edu/genome_software/other/primer3.html). XIPHF is located 889-870 and XIPHR is located 369-388 relative to *Caenorhabditis elegans* (accession number X03680).

template purified DNA. The DNA was sequenced in-house using an ABI 377 DNA sequencer. Thereafter, sequence information was assembled and edited using Sequence Navigator Software (Applied Biosystems, Warrington, UK).

Multiple alignment and phylogenetic analysis: CLUSTAL X v. 1.81 (Thompson et al., 1997) was used to generate multiple alignments with default settings. Thereafter, manual editing was done using GeneDoc (http://www.psc.edu/biomed/genedoc/). Columns with more than 50% gaps were removed. *Mononchus aquaticus* (GenBank accession number AY297821) was chosen as the outgroup for the analysis as Mononchidae is within clade I, closely related to Dorylaimida (Blaxter et al., 1998). The choice of out-group will allow comparability with future studies on Longidoridae.

Phylogenetic analysis was done using TREE-PUZZLE (Strimmer and von Haeseler, 1996) and programs from the PHYLIP package (Felsenstein and Churchill, 1996). For the rDNA analysis, TREE-PUZZLE estimated the following parameters: expected transition/transversion ratio (Ts/Tv) and alpha shape parameter for an F84 plus Gamma rates model. A maximum likelihood tree was then estimated (using Ts/Tv set at 1.75 and alpha set at 0.17) using the PHYLIP DNAML program. Bootstrap analysis was done with 100 replicates using the PHYLIP SEQBOOT and CONSENSE programs.

A phylogenetic tree (observed morphometric tree) was calculated based on standard nematological morphometric measurements. The 11 characters in the morphometric data set (total body length, odontostyle lenght, odontophore length, spear length, tail length, body width at anus, maximum body width, V% and a, c and c′ ratios) were transformed to have the same mean and variance. A maximum likelihood tree was then estimated using the PHYLIP CONTML program. Bootstrap analysis was done with 100 replicates using a SEQBOOT-like program written locally and the PHYLIP CONSENSE program. The heuristic searches done by DNAML and CONTML both used the global rearrangements option (equivalent to SPR heuristic search).

The topologies of the morphometric and 18S rDNA trees were compared using the Kishino Hasegawa, (KH) test (Kishino and Hasegawa, 1989). A hypothetical (expected) morphometric tree was generated based on the results of the rDNA analysis. The KH test was then used to compare the observed morphometric tree with the expected tree using the PHYLIP CONTML program.

Results

Identical 18 rDNA sequences were obtained from individuals from each population of the same species with two exceptions. Three of the four individual females of the *Xiphinema setariae/vulgare* complex studied were identical; however, when compared to a fourth female, 7 single nucleotide substitutions (0.4%) were apparent. Also, two morphotypes of *X. krugi* from different populations, morphologically distinguished from each other mainly by tail shape, had 2.0% sequence divergence, corresponding to 35 substitutions.

Within the *X. americanum* group, nearly identical sequences were obtained among the studied putative taxonomic species, with interspecific divergence ranging from only 1 to 3 substitutions. The 18S sequence of *X. americanum sensu stricto* from specimens originating from South Africa (provided by F. Lamberti, CNR, Bari, Italy) was identical to that of *X. peruvianum;* however, *X. americanum sensu stricto* was not included in the phylogenetic analysis as no comparative morphometric data were available.

The sequence determined for the 18S rDNA of the *Xiphinema* and *Xiphidorus* species studied varied in length, ranging from 1757 bp (*Xiphinema ensiculiferum* and *Xiphidorus yepesara yepesara*) to 1780 bp (*X setariae/vulgare*). After trimming excess nucleotides at both the 5′ and 3′ ends to effect a common starting and end point, a multiple sequence alignment of all trimmed 18S rDNA sequences, including the outgroup, yielded a consensus length of 1762 bp, of which 1401 (79.5% of all sites) were constant. Estimated average nucleotide frequencies among the studied longidorids were similar: 24.6% (T), 27.9% (A), 26.1% (G), and 21.4% (C).

The phylogenetic tree (Fig. 1) indicated that species from the genera *Xiphinema* and *Xiphidorus* were separated into distinct groups, confirming their known taxonomic status within Longidoridae. The maximum likelihood consensus tree yielded three distinct clades comprising (i) *Xiphidorus* species (*X. yepesara yepesara, X. yepesara parthenus, X. minor, X. balcarceanus,* and an undescribed *Xiphidorus* species), (ii) species belonging to the *X. americanum* group (*X. brevicolle, X. diffusum, X. oxycaudatum,* and *X. peruvianum*), and (iii) all other non *X. americanum* group species.

Within the cluster representing the *Xiphidorus* spe-

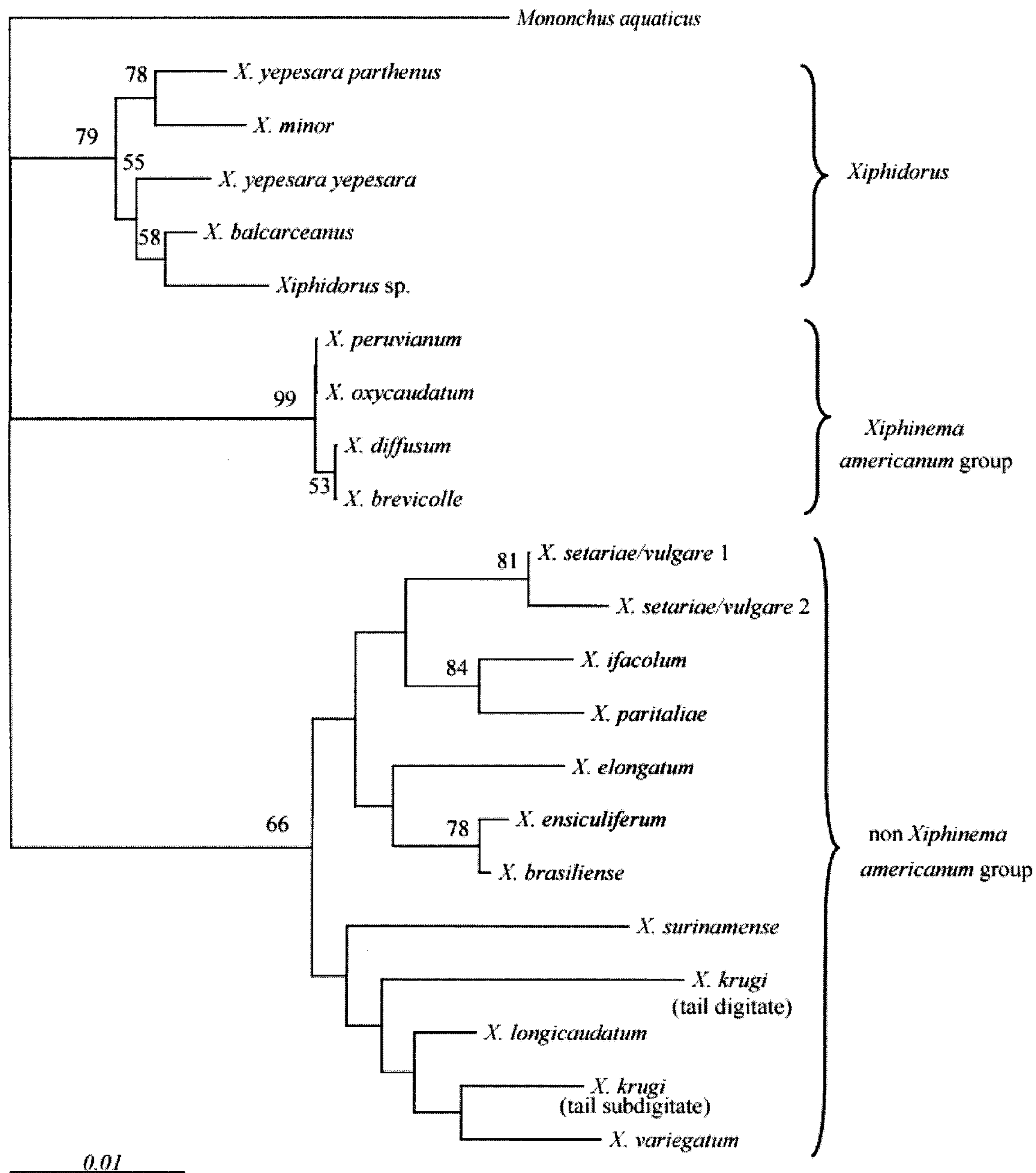

FIG. 1. Phylogenetic tree showing relationships between *Xiphinema* and *Xiphidorus* species based on sequences of 18S rDNA. The tree was constructed using DNAML. The numbers indicate the bootstrap values higher than 50. Branch lengths are drawn to be proportional to the number of changes inferred. The out-group branch length was reduced by 90% to clarify the relationships within the tree.

cies, *X. yepesara parthenus* and *X. minor* formed a subgroup outwith the other *Xiphidorus* species. Also, *X. yepesara yepesara* was separated from the remaining two species.

Relationships between the non *X. americanum* group species were poorly resolved because there was little statistical support to clearly separate species; however, three subgroups, comprising (i) the *X. setariae/ vulgare* complex, (ii) *X. ifacolum* and *X. paritaliae,* and (iii) *X. brasiliense* and *X. ensiculiferum,* were well resolved.

The observed morphometric tree confirmed the same three main clades obtained in the 18S rDNA tree with a few exceptions (Fig. 2). In the observed morphometric tree, *Xiphidorus minor* and *Xiphinema elongatum* were included with the *X. americanum* group species and *X. ifacolum* clustered with four *Xiphidorus* species (Fig. 2). Topologies, based on clade position, of both the observed and expected, derived from the 18S tree (expected tree not shown), morphometric trees were not significantly different as indicated by the Kishino Hasegawa (KH) test.

Discussion

The 18S rDNA sequences provided useful information for constructing a phylogeny of *Xiphinema*

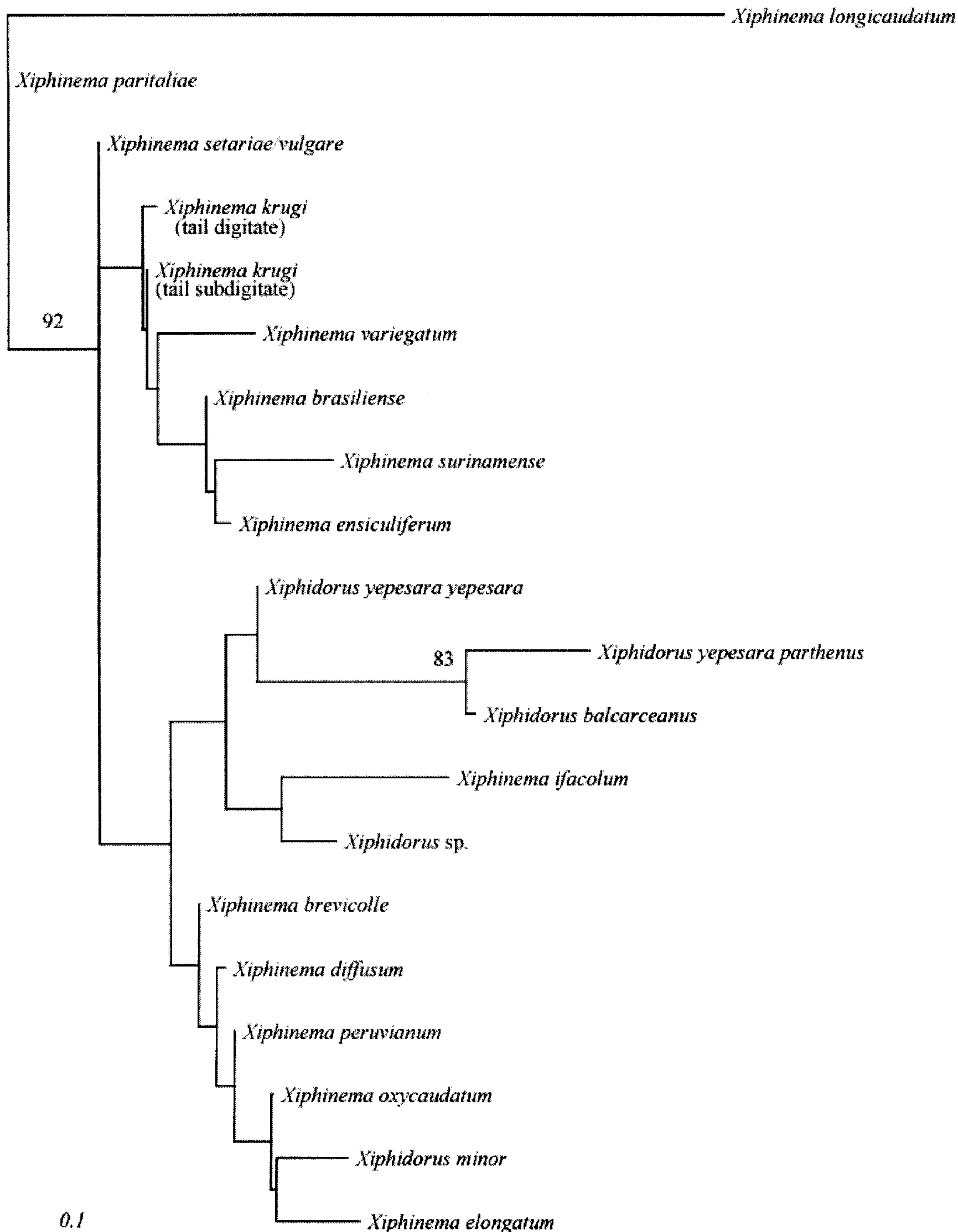

FIG. 2. Observed morphometric phylogenetic tree showing relationships between *Xiphinema* and *Xiphidorus* species based on 11 morphometric characters (Oliveira et al., 2003). The tree was constructed using CONTML. The numbers indicate the bootstrap values higher than 50. Branch lengths are drawn to be proportional to the number of changes inferred.

and *Xiphidorus* species, primarily at the genus level. Several studies have used 18S rDNA sequences to successfully assess the phylogenetic relationship among different nematode taxa, principally at the family level (Aleshin et al., 1998; Blaxter et al., 1998; Kampfer et al., 1998) rather than closely related species (Fitch et al., 1995; Kanzaki and Futai, 2002). Moreover, the 5′ segment of the 18S rDNA has recently been utilized to develop a simplified molecular system that permits an estimation of the diversity of soil and marine nematodes using molecular operational taxonomic units (MOTU) (Blaxter et al., 2002; Floyd et al., 2002).

Within *Xiphidorus,* our molecular analysis affirmed the monophyly of this genus. Furthermore, our data

indicated that this clade is well resolved. In addition, significant divergence was found between *X. yepesara yepesara* and *X. yepesara parthenus.* Based on this information and a multivariate statistical analysis of 11 morphometric characters (Oliveira et al., 2003), a taxonomic re-appraisal may be required to clearly define whether they are morphological synonyms (Chaves et al., 1999), sub-species (Decraemer et al., 1996), or distinct taxonomic species as originally described (Monteiro, 1976; Monteiro et al., 1981).

In our study it was observed that species belonging to the *X. americanum* group formed a single group separated from the other *Xiphinema* species. As with previous taxonomic studies (Lamberti et al., 2000) that noted only minor morphological differences among the 51 putative *X. americanum* group species, it was evident based on 18S rDNA sequences that it was also difficult to separate these species, perhaps suggesting that instead of putative species, the *X. americanum* group represents numerous morphotypes with large inter- and intra- population variability resulting from environmentally driven morphometric plasticity (Arpin, 2001; Arpin et al., 1988; Brown, 1985; Doucet et al., 1996). A more detailed study of this group using molecular markers is in progress (Zhang et al., unpubl. data).

Within the cluster representing non *X. americanum* group species, where there was little statistical support to clearly separate species, only three subgroups, comprising (i) the *X. setariae/vulgare* complex, (ii) *X. ifacolum* and *X. paritaliae,* and (iii) *X. brasiliense* and *X. ensiculiferum,* were apparent.

Although *X. ifacolum* and *X. paritaliae* are morphologically distinguished from each other, they have some common taxonomic characteristics. In both species, the females have two genital branches with the presence of uterine differentiation (Z-organ). In *X. ifacolum* the Z-organ is obvious whereas in *X. paritaliae* it is lightly sclerotized. Also, in approximately 40% of the *X. paritaliae* females studied by Ferraz (1980), the inner surface of the cuticle of the tail tip formed a thin and short blind canal, which was similar to that of *X. ifacolum.* Thus the grouping of *X. ifacolum* and *X. paritaliae* suggested by molecular phylogenetic analysis could be expected. Further, for these two species, our molecular phylogenetic analysis concurs with a classical phylogenetic study based on morphological characters proposed by Coomans et al. (2001), where they belonged to the same cluster.

Similarly, the known taxonomic relationship of *X. brasiliense* and *X. ensiculiferum* was strongly supported in this analysis. Both species are monodelphic with an anteriorly positioned vulva and the anterior branch of the reproductive system absent.

Only two species, the *X. setariae/vulgare* complex and *X. krugi,* exhibited very small levels of possible intraspecific divergence of the 18S rDNA, 0.4% and 2.0%, respectively. However, the variability between the two *X. krugi* populations may be indicative that these are in fact two different species. This concurs with Oliveira et al. (2003), who noted morphological and morphometric differences between these populations. With regard to the limited variation in the *X. setariae/vulgare* complex sequence, it is perhaps more likely that the variability was due to sequence errors ascribed to PCR artefacts.

Our data suggest that 18S rDNA sequences appear not to be a useful marker to discriminate *Xiphinema* at the species level. However, there is evidence that they do have sufficient resolution to separate *X. americanum* group morphotypes from non *X. americanum* species, thus raising the possibility for the requirement of a taxonomic revaluation of the *X. americanum* group.

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