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Ribosomal and Mitochondrial DNA Analyses of *Xiphinema americanum*-Group Populations

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Abstract: The 18S ribosomal DNA (rDNA) and cytochrome oxidase I region of mitochondrial DNA (mtDNA) were sequenced for 24 *Xiphinema americanum*-group populations sourced from a number of geographically disparate locations. Sequences were subjected to phylogenetic analysis and compared. 18S rDNA strongly suggested that only *X. pachtaicum*, *X. simile* (two populations) and a *X. americanum s.l.* population from Portugal were different from the other 20 populations studied, whereas mtDNA indicated some heterogeneity between populations. Phylogenetically, based on mtDNA, an apparent dichotomy existed amongst *X. americanum*-group populations from North America and those from Asia, South America and Oceania. Analyses of 18S rDNA and mtDNA sequences underpin the classical taxonomic issues of the *X. americanum*-group and cast doubt on the degree of speciation within the *X. americanum*-group.

Key words: 18S rDNA, longidorid, Longidoridae, molecular analysis, mtDNA, nematode, taxonomy.

The taxonomy of the *Xiphinema americanum*-group is controversial, comprising either 34 (Luc et al., 1998), 38 (Coomans et al., 2001) or 50 (Barsi and Lamberti, 2004; Lamberti et al., 2004) putative species, depending on the taxonomic authority. For example, Luc et al. (1998) proposed that *X. diffusum*, *X. incognitum* and *X. taylori* were junior synonyms of *X. brevicollum*. Lamberti et al. (2000) heavily criticized the delineation and definition of the *X. americanum*-group used by Luc et al. (1998). In turn, Luc and Baujard (2001) heavily criticized Lamberti et al. (2000). However, a hierarchical tree analysis based on morphometrics of 117 *X. americanum*-group populations indicated that *X. brevicollum*, *X. diffusum*, *X. incognitum* and *X. taylori* could to varying degrees separate the species (Lamberti et al., 2002). Recently, using molecular techniques, Oliveira et al. (2005) unequivocally demonstrated that *X. brevicolle* (note, this is the species name used by Oliveira et al., 2005) and *X. diffusum* were indeed distinct species. In order to try and unravel these taxonomic conundrums, we have taken an expansive view and without a priori prejudice considered the maximum number of potential species notwithstanding synonymies. It is far easier to discover potential species to synonymize by employing an expansive view than to delineate species based on a conservative species list which would arguably be subsequently considered instead as an intra-specific variability.

Species belonging to the *X. americanum*-group have a cosmopolitan distribution, with several in the Americas and Japan being of particular economic importance, as they are natural virus-vectors of four members of the genus *Nepovirus* (Taylor and Brown, 1997). Biologically, some species reported from Africa, Europe and the US have been shown to have only three and not the usual four juvenile developmental stages (JDS; Halbrendt and Brown, 1992; Coomans and Heyns, 1997; Barsi and Lamberti, 2002). Recognizing the complex taxonomic issues surrounding the *X. americanum*-group, Lamberti et al. (2002) recommended the use of molecular tools to discriminate putative *X. americanum* species and/or populations.

A plethora of recent phylogenetic studies on plant-parasitic nematodes (including the Longidoridae) based on ribosomal DNA (rDNA) have been published (for example, Subbotin et al., 2001; De Ley et al., 2002; Boutsika et al., 2004a, 2004b; De Luca et al., 2004; Ferris et al., 2004; Neilson et al., 2004; Oliveira et al., 2004a, 2004b; Subbotin et al., 2004; Ye et al., 2004), with some authors utilizing rDNA to make definitive taxonomic statements on specific species either with (Subbotin et al., 2002; Oliveira et al., 2004a) or without (Boutsika et al., 2004b) supporting morphological data.

Oliveira et al. (2004b) reported that four putative species (*X. brevicolle*, *X. diffusum*, *X. peruvianum* and *X. oxycaudatum*) of the *X. americanum*-group had high 18S rDNA sequence homology. Furthermore, these four species separated from other *Xiphinema* species with strong statistical support, leading the authors to suggest that a critical taxonomic re-evaluation of the *X. americanum*-group was required. Similarly, He (2003) noted high sequence homology in the D2/D3 expansion region of rDNA amongst the majority of the 17 putative species of the *X. americanum*-group studied, although a dichotomy of the *X. americanum*-group was apparent, with members of the *X. pachtaicum* sub-group (sensu Lamberti and Ciancio, 1993) separated from the other *X. americanum*-group species.

Although mitochondrial DNA (mtDNA) evolves very quickly in nematodes (Moritz et al., 1987; Blouin et al., 1998; Denver et al., 2000), studies that have utilized mtDNA as a means of discriminating plant-parasitic spe-

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TABLE 1. Populations of *Xiphinema americanum*-group nematodes used to sequence 18S rDNA and mtDNA.

Nematode species/population	Country of origin	Plant host	Collector	Accession number	
				18S rDNA	mtDNA
Xiphinema americanum s.str.	South Africa	*Vitis* sp.	F. Lamberti	AM086684	AM086690
Xiphinema americanum s.l.	Portugal	Coniferous forest	M. T. M. Almeida	AM086679	AM086709
Xiphinema americanum s.l.	Nepal	*Carica papaya*	N. Pokharel	AM086683	AM086704
Xiphinema americanum s.l.	USA	Orchard fruits	F. Lamberti	AM086671	AM086689
Xiphinema americanum s.l.	USA	Orchard fruits	F. Lamberti	AM086672	AM086691
Xiphinema brevicolle[a]	Brazil	*Coffea arabica*	C. M. G. Oliveira	AY297822	AM086707
Xiphinema citricolum	USA	Orchard fruits	F. Lamberti	AM086686	AM086693
Xiphinema diffusum[a]	Australia	*Vitis* sp.	F. Lamberti	AM086685	AM086700
Xiphinema diffusum[a]	Brazil	*Prunus persica*	C. M. G. Oliveira	AY297823	AM086698
Xiphinema diffusum[a]	Brazil	Unknown	C. M. G. Oliveira	AM086677	AM086699
Xiphinema diffusum[a]	China	Unknown	Q. Lui	AM086669	AM086701
Xiphinema incognitum[a]	China	*Litchi chinensis*	J. Zheng	AM086678	AM086705
Xiphinema incognitum[a]	China	*Litchi chinensis*	J. Zheng	AM086670	AM086706
Xiphinema floridae	USA	Orchard fruits	F. Lamberti	AM086687	AM086696
Xiphinema georgianum	USA	Orchard fruits	F. Lamberti	AM086688	AM086695
Xiphinema pachtaicum	Slovakia	*Juglans regia*	M. Liskova	AM086682	AM086710
Xiphinema peruvianum	Brazil	*C. arabica*	C. M. G. Oliveira	AY297832	AM086712
Xiphinema peruvianum	USA	Orchard fruits	F. Lamberti	AM086674	AM086692
Xiphinema rivesi	USA	Orchard fruits	R. Robbins	AM086673	AM086697
Xiphinema simile	Slovakia	*Vitis vinifera*	M. Liskova	AM086680	AM086708
Xiphinema simile	Serbia	Unknown	L. Barsi	AM086681	AM086711
Xiphinema tarjanense	USA	Orchard fruits	F. Lamberti	AY283170	AM086694
Xiphinema taylori[a]	Slovakia	*J. regia*	M. Liskova	AM086675	AM086702
Xiphinema taylori[a]	Slovakia	*Cerasus* sp.	M. Liskova	AM086676	AM086703

[a] syn. *X. brevicollum* after Luc et al. (1998).

cies have been primarily restricted to *Meloidogyne* (Powers et al., 1986; Hugall et al., 1994, 1997; Stanton et al., 1997; Blok et al., 2002). Here we compare the utility of 18S rDNA and mtDNA to discriminate *X. americanum*-group populations.

MATERIALS AND METHODS

Nematodes: Twenty-four *X. americanum*-group populations (Table 1) were collected from a number of geographically disparate locations. Specimens were extracted from soil using standard techniques, placed into 1M NaCl, sent to the Scottish Crop Research Institute and frozen at –20°C until DNA extraction.

DNA extraction, PCR amplification and sequencing: DNA extraction methodology, 18S rDNA purification, sequencing and the relevant PCR conditions are all described by Oliveira et al. (2004b). A minimum of 3 nematodes from each population were sequenced. Sequences for cytochrome oxidase I (COI) were obtained using the forward CO1F primer in combination with different reverse primers (Table 2). Primers CO1F and CO1R were designed using PRIMER3 software (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) from nematode mtDNA sequences publicly available from GenBank. Our area of investigation within the COI gene was approximately between nt 9,717 and nt 10,119 based on the mtDNA genome of *X. americanum* (He et al., 2005; Genbank Accession: AY382608). The remaining primers were designed from *X. americanum*-group mtDNA sequences obtained during this study. In general, PCR conditions were as follows but were not optimal for all species/populations tested: 1 cycle of 94°C for 1 min, 50°C for a further 1 min and 72°C for 2 min. This was followed by 40 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min. The PCR was completed with a final extension phase of 94°C for 1 min, 45°C for 1 min and 72°C for 5 min. For mtDNA, the reaction mix comprised a single PCR PureTaq Ready to go Bead (GE Bioscience, UK), 23 µl sterile water, 1 µl template DNA and 0.5 µl of each 10 µM primer. Purified DNA was sequenced directly in both directions using a Big Dye Terminator cycle sequencing kit v3.1 (Applied Biosystems, Warrington, UK), according to the instructions listed by the manufacturer. For each sequencing reaction, the following reagents were added to a 0.5-ml microcentrifuge tube: 4 µl terminator Ready Reaction Mix, 1 µl primer (3.4 µM) and 5 µl template purified DNA. DNA was sequenced in-house using an ABI 377 DNA sequencer. The quality of the sequences produced was checked

TABLE 2. Primers used to characterize the cytochrome oxidase I of mitochondrial DNA from populations of *Xiphinema americanum*-group.

Primer code	Direction	Sequence (5′–3′)
CO1F	Forward	GATTTTTTGGKCATCCWGARG
CO1R	Reverse	CWACATAATAAGTATCATG
R2	Reverse	GTMACACCCGTRAAAATMCCAAA
XIPHR1	Reverse	ACAATTCCAGTTAATCCTCCTACC
XIPHR2	Reverse	GTACATAATGAAAATGTGCCAC

using Sequence Navigator Software (Applied Biosystems, Warrington, UK).

Data analysis: CLUSTAL X v.1.81 (Thompson et al., 1997) was used to generate multiple sequence alignments with default settings for both 18S rDNA and mtDNA sequences. Thereafter, trimming excess nucleotides at the 5′ and 3′ ends to effect a common starting and end point was done using GeneDoc (Nicholas et al., 1997).

MODELTEST (Posada and Crandall, 1998) estimated that a F84 plus gamma + invariant rate heterogeneity model was the most appropriate for the dataset under study.

Phylogenetic analysis was carried out using the Maximum Likelihood (ML) approach. ML was preferred to Maximum Parsimony (MP) because ML uses: i) all columns of the multiple sequence alignment (i.e., not just phylogenetically informative columns) and ii) information on branch lengths when evaluating trees (Swofford et al., 1996). ML trees were estimated (Ts/Tv set at 1.87 and alpha at 0.02 for the 18S rDNA alignment; Ts/Tv set at 3.63 and alpha at 0.28 for the mtDNA alignment) using the PHYLIP v.3.6a DNAML sub-routine (Felsenstein and Churchill, 1996). Furthermore, ML was preferred over Bayesian analysis, as the latter method is recognized to overestimate support for tree topology (Suzuki et al., 2002; Simmons et al., 2004). Sequence data for *Californidorus* sp. (AY283155) was used as the outgroup for the 18S rDNA analysis, and mtDNA sequences for *Caenorhabditis elegans* (AY268112), *Scutellonema bradys* (AY268114) and *Meloidogyne hapla* (AY268113) were used as the outgroups for the mtDNA analysis.

Results

A phylogenetic analysis based on 18S rDNA sequences yielded well resolved groups (Fig. 1). The putative species *X. pachtaicum* was distinct from all species/populations, and *X. simile* (two populations) and *X. americanum* sensu lato (Portugal) were distinct from the remaining populations of *X. americanum*-group populations studied (Fig. 1). In contrast, the remaining putative *X. americanum*-group species/populations had sequence homology of >99%, as reflected in their close phylogenetic relationship (Fig. 1). A consensus sequence of 1,752 bp was produced, of which 1,623 bp, i.e., 92.6% of all nucleotides, were constant. The estimated average nucleotide frequencies among the *X. americanum*-group populations were relatively similar, being 24.8% A, 25.9% C, 21.6% G and 27.7% T. Populations identified using classical morphological techniques as *X. diffusum* (syn. *X. brevicollum* after Luc et al., 1998) from China had 18S rDNA sequences identical to those identified as *X. diffusum* and *X. taylori* (syn. *X. brevicollum*) from Brazil and Slovakia, respectively. The majority of *X. americanum*-group specimens from North America had sequence divergence of <0.5% and were separated from a group comprising the majority of populations from Asia, South America and Oceania.

In contrast to 18S rDNA, mtDNA was more heterogeneous, with only 136 bp from a consensus of 405 bp (33.6%) constant. The estimated average nucleotide frequencies were AT-rich, comprising 25.5% A, 14.0% C, 22.4% G and 38.1% T. Furthermore, mtDNA sequences representing *X. diffusum* (Brazilian populations only) and *X. taylori* were within each species identical. *Xiphinema americanum* sensu lato (Portugal), *X. pachtaicum* and both *X. simile* populations formed a well resolved group distinct (Fig. 2) from the other species/populations, thus concurring with the 18S rDNA analysis (Fig. 1), with *X. simile* (Slovakian population) being an outlier. The dichotomy between *X. americanum*-group populations from North America and those from Asia, South America and Oceania was also apparent for mtDNA, although the groupings were less well resolved than those produced by 18S rDNA (Figs. 1,2). No intrapopulation variability in the mtDNA was detected.

Discussion

Ever since the inception of the nomenclature "*Xiphinema americanum*-group" by Tarjan (1969) to encompass morphological and morphometric variability of *X. americanum* populations, controversy has surrounded their degree of speciation (Luc et al., 1998; Coomans et al., 2001; Lamberti et al., 2004).

The putative species within the *X. americanum*-group are characterized by minimal inter- and intraspecific variation in both morphological and morphometric characters and, consequently, Loof and Luc (1990) excluded them from their polytomous key of *Xiphinema*. Subsequently, polytomous keys for the group have been published (Lamberti et al., 2000, 2004), but both keys contain errors that compromise identification of a number of species. Such taxonomic pedantry would normally be irrelevant, but unfortunately a few of the *X. americanum*-group species are known vectors of *Nepovirus* (Taylor and Brown, 1997), with the concomitant effect that *X. americanum* is a quarantined organism in the European Union (http://www.eppo.org/QUARANTINE/listA1.htm); hence correct species identification is imperative within a phytosanitary context.

Consistent with the global tree-of-life project (Stoeckler, 2003), NemATOL (http://nematol.unh.edu) utilizes 18S rDNA as the de facto standard ribosomal region to molecularly characterize nematodes. In this study, with a few exceptions, 18S rDNA was homogeneous for the *X. americanum*-group populations studied and could not discriminate putative species that were raised according to minute differences in morphology and morphometrics. It is possible that this lack of discriminatory power was due to insufficient evolutionary change occurring in the 18S rDNA to provide useful

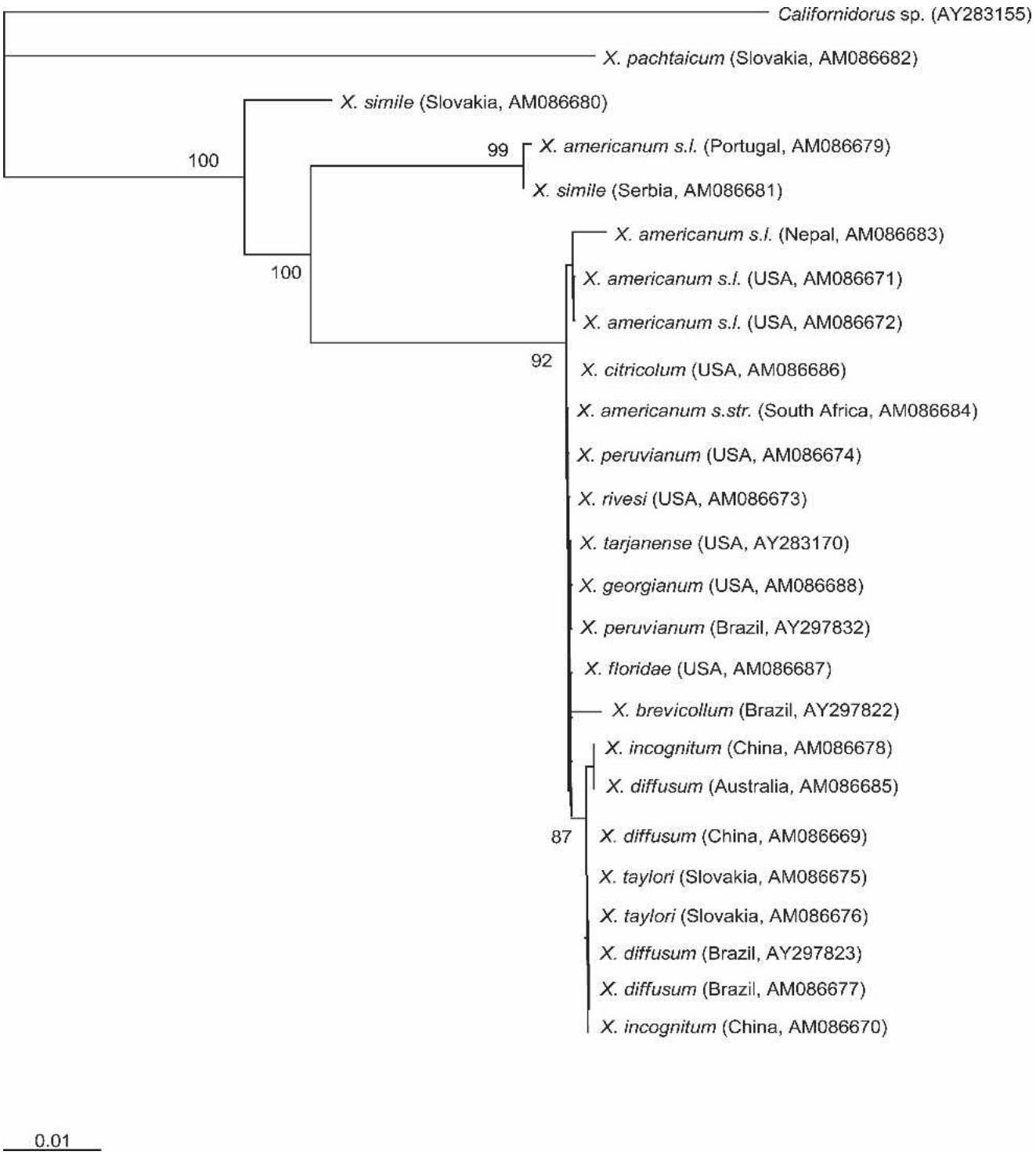

FIG. 1. Phylogenetic tree showing relationships between *Xiphinema americanum*-group populations based on sequences of 18S rDNA. The numbers indicate the bootstrap values higher than 50. Branch lengths are drawn to be proportional to the number of changes inferred.

phylogenetic information. Similarly, with the same exceptions as 18S rDNA, mtDNA did not resolve what is currently perceived to be extant *X. americanum*-group species, even though mtDNA evolves quickly in nematodes (Moritz et al., 1987; Blouin et al., 1998; Denver et al., 2000). However, analysis of both 18S rDNA and mtDNA indicated that the *X. pachtaicum*, *X. simile* and *X. americanum s.l.* (from Portugal) were different from all other species/populations tested. Both *X. pachtaicum* and *X. simile* belong to the "*X. pachtaicum* group" (Lamberti and Ciancio, 1993), and this separation from other members of the *X. americanum*-group is consistent with multivariate and cluster analyses of morphological data (Lamberti and Ciancio, 1993; Lamberti et al.,

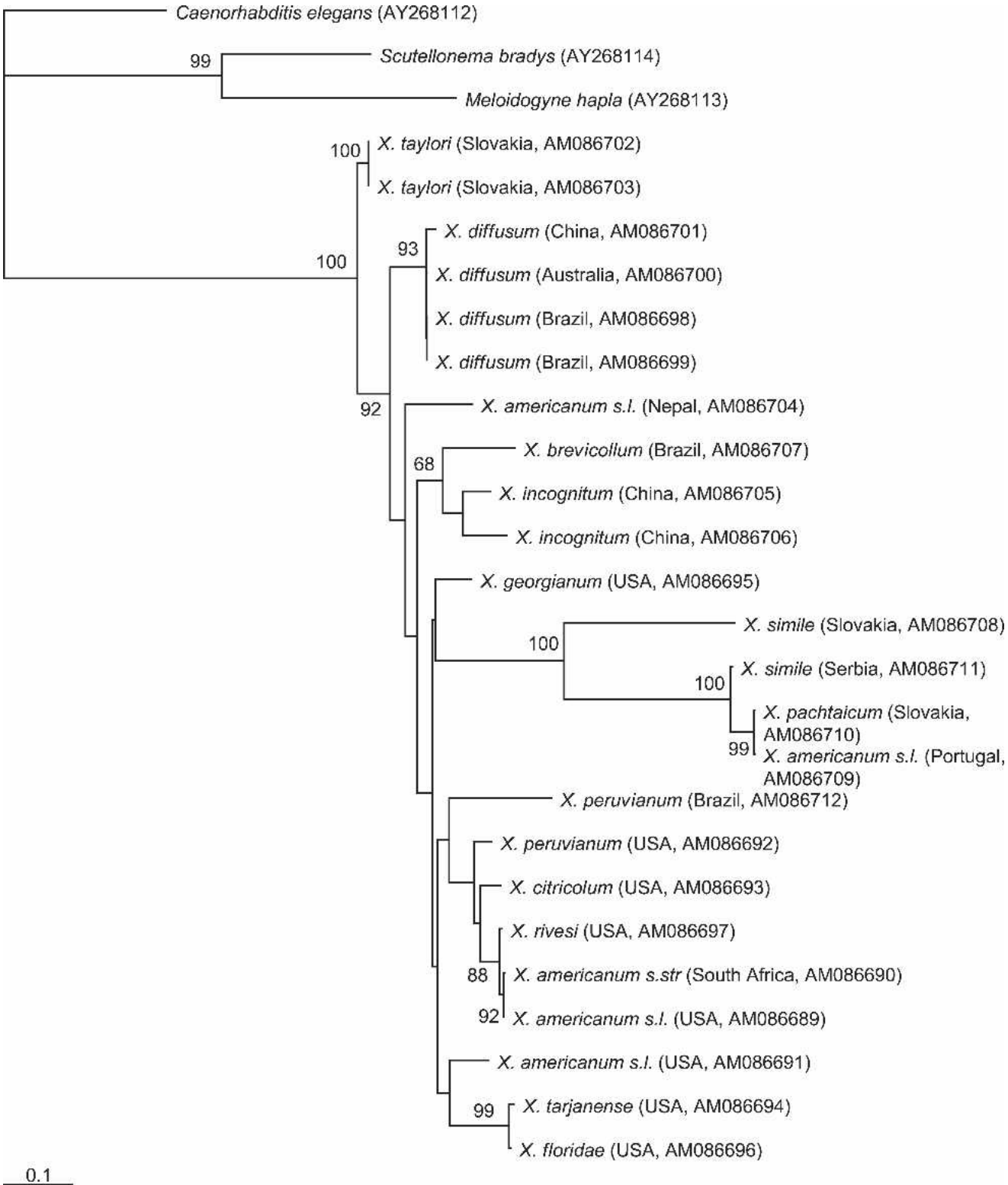

FIG. 2. Phylogenetic tree showing relationships between *Xiphinema americanum*-group populations based on sequences of cytochrome oxidase I mtDNA. The numbers indicate the bootstrap values higher than 50. Branch lengths are drawn to be proportional to the number of changes inferred.

2002). Similarly, the dichotomy of putative species such as *X. diffusum* and *X. taylori* from species originating from North America is also consistent with that reported by Lamberti and Ciancio (1993) and Lamberti et al. (2002). Recently, Oliveira et al. (2005) published species-specific diagnostic primers that discriminate between *X. brevicolle* (syn. *X. brevicollum*) and *X. diffusum*, and here our analyses separated the two species into

different groups, thus supporting Lamberti et al. (1991, 2002).

In this study, the degree of 18S rDNA sequence divergence (<0.5%) for the *X. americanum*-group populations originating from North America is similar to intraspecific divergence reported for *Longidorus diadecturus* (0.6%; Neilson et al., 2004) and *X. setariae/vulgare* complex (0.4%; Oliveira et al., 2004b). Thus, it could be suggested that extant species such as *X. citricolum*, *X. floridae*, *X. georgianum*, *X. peruvianum* and *X. americanum* sensu stricto are merely intraspecific variants of a single species. However, this result should be viewed with caution, as the classical taxonomy of the group is so problematic it is possible that incorrect identifications were made, which in turn would have a concomitant effect on the interpretation of the sequence data; thus, a self-perpetuating scenario ensues. To compound the interpretation further, intraspecific variability of 18S rDNA has to date been largely ignored by nematologists.

Similarly, there are few published data on intraspecific variability of mtDNA in nematodes. Blouin et al. (1998) noted that for the ruminant parasite *Ostertagia ostertagi* the maximum sequence divergence observed between individuals of the same interbreeding population was 6%. The same authors also stated that mtDNA sequence divergence between closely related species is typically in the range of 10 to 20%. Thus, taking a conservative approach in this study and using 20% mtDNA sequence divergence as a crude measure to determine conspecificity (assuming the classical taxonomy is accurate) would suggest that *X. georgianum* and *X. peruvianum* may be valid species, whereas the other populations from North America appear to be examples of intraspecific variability of a single species or perhaps represent cryptic species (Blouin et al. 1998; Blouin, 2002).

In conclusion, analyses of both 18S rDNA and mtDNA sequences have underpinned the taxonomic issues of the *X. americanum*-group. It is clear that a thorough and combined morphological and molecular investigation is required to unravel the complexities of the *X. americanum*-group. Such an approach was taken to clarify taxonomic questions surrounding *Xiphidorus* (Oliveira et al., 2004a). Multiloci sequence typing (MLST), where a number (typically >7) of molecular targets are sequenced (Enright and Spratt, 1998; Maiden et al., 1998), is already used to elucidate taxonomic information of closely related bacterial sequences, and this may be an useful approach for future taxonomic analysis of this complex nematode group.

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