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Xiphinema krugi, Species Complex or Complex of Cryptic Species?

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Abstract: Fourteen morphologically putative populations of *X. krugi* were clearly separated into four different profiles by RFLP analysis (Alu I and Hinf I), sequencing of the ITS-1 region, and subsequent Maximum Likelihood phylogenetic analyses. These four profiles were further supported by a principal component analysis of morphometric characters that yielded four taxonomic clusters matching those produced by the molecular data. Sequence homology was greater amongst populations that represented the same RFLP profile than between profiles and similar both between representative populations of the RFLP profiles and putative closely related *Xiphinema* species. This study suggests that *X. krugi* is a potential species complex comprised of at least four distinct genotypes.

Key words: Brazil, DNA sequence, intraspecific variability, longidorids, speciation, *Xiphinema*

Xiphinema krugi is a pseudomonodelphic species originally described from the rhizosphere of natural vegetation in Piracicaba, São Paulo State, Brazil (Lordello, 1955). It is widespread throughout Brazil (Loof and Sharma, 1979; Ferraz, 1980; Rashid et al., 1986; Ferraz et al., 1989; Germani, 1989; Costa Manso et al., 1994; Maximiniano et al., 1998) and was the most prevalent *Xiphinema* species recorded during a recent national survey of Brazil (Oliveira et al., 2003). *Xiphinema krugi* was considered by Coomans et al. (2001) pantropical in distribution. However, with the exception of Europe and Antarctica, it has been reported from all continents (e.g., Frederick and Tarjan, 1974; Heyns, 1977; Luc and Hunt, 1978; Razak and Loof, 1989; Robbins and Brown, 1991). In Latin America apart from Brazil, *X. krugi* has been reported from the following countries: Argentina (Luc and Doucet, 1990; Decraemer et al., 1998), Colombia (Volcy, 1990), Martinique (Luc and Coomans, 1992), Paraguay (Luc and Hunt, 1978), Surinam (Loof and Maas, 1972), Trinidad (Bala, 1984), Uruguay (s'Jacob and Loof, 1996), and Venezuela (Petit and Crozzoli, 1995; Crozzoli et al., 2001).

As a result of interpopulation morphological and morphometric heterogeneity, the taxonomic status of *X. krugi* has been questioned (Frederick and Tarjan, 1974; Lamberti and Tarjan, 1974; Luc and Hunt, 1978; Ferraz, 1980). Lamberti and Tarjan (1974) confirmed the synonymization of both *X. denoudeni* (Loof and Maas, 1972) and *X. loosi* (Southey and Luc, 1973) with *X. krugi* based on the similar structure of the female reproductive system and a subdigitate tail shape.

Luc and Hunt (1978) examined the tail shape and structure of the anterior female genital branch of *X. krugi* populations, *X. denoudeni* and *X. loosi* both from disparate geographical areas and concurred with the previous synonymization. However, close examination of their data (Table 3 and Figure 6 in Luc and Hunt, 1978) revealed potential areas of doubt that question the validity of the conclusions made by those authors. Firstly, the type *X. krugi* population was excluded from the analysis, thus removing the possibility of any definitive statement to be made. Secondly, it is clear from Figure 6 (Luc and Hunt, 1978) that there are four clusters, one of which formed a discrete group comprised of populations previously described as *X. loosi*, with short tail length (mean = 29.3 µm), hemispheroid tail shape and a short anterior genital branch (60.5 µm). A second group, represented by a single population from Florida (USA), was separated by having a longer tail (44.5 µm) and being conoid in shape with a distinct ventral peg. The third group, comprised of populations from Paraguay and Surinam (pop. 4 and 6; Luc and Hunt, 1978), had a mean tail length of 35 µm and an anterior genital branch of 72 µm. The remaining group, comprised of the putative *X. krugi* (Lordello, 1955) populations, had a mean tail length of 31.8 µm and a longer anterior genital branch (97.5 µm).

An examination of more than 300 *X. krugi* females from populations collected in eight Brazilian states (Ferraz, 1980) also noted considerable variability in tail shape. The majority (approximately 90%) of the specimens had a characteristic ventral tail peg, whilst the remainder were hemispheroidal.

Based on tail shape and a principal component analysis of 11 morphometric characters (Oliveira et al., 2003), two putative morphotypes of *X. krugi* were noted (populations PX37b and PX59a). Interpopulation divergence (2.0%) of their respective 18S rDNA was also noted (Oliveira et al., 2004b).

The reported variability in *X. krugi* (Frederick and Tarjan, 1974; Lamberti and Tarjan, 1974; Luc and Hunt, 1978; Ferraz, 1980; Oliveira et al., 2003, 2004b) strongly suggested the possibility of a species complex. Therefore, the objective of the present study was to screen 14 *X. krugi* populations using a combined classical taxonomic and molecular approach to determine if it was possible to characterize different *X. krugi* morphotypes.

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Materials and Methods

Nematodes: Soil samples were collected from different habitats and vegetation types, including crop plants, grass-land, savannah (cerrado), Amazonian forest vegetation, and natural habitats from all five regions of Brazil (Fig. 1). Each pooled sample consisted of approximately four 1.5 to 2.0 kg samples of moist soil collected around actively growing young roots, to a depth of 40 cm. The soil was placed in a polythene bag, stored in a cooled box (15–20°C) and immediately transported to the laboratory. *Xiphinema krugi* was recorded from the Brazilian States of Amapá (AM), Mato Grosso (MT), Mato Grosso do Sul (MS), Minas Gerais (MG), Parana (PR), Rio Grande do Sul (RS), Santa Catarina (SC), and São Paulo (SP) (Fig. 1).

DNA extraction: DNA was extracted from a minimum of two individual females using a NaOH lysis protocol from 14 populations (Table 1) as described by Oliveira et al. (2004b).

DNA was also included from *X. longicaudatum*, *X. surinamense*, and *X. variegatum*, which are closely related *Xiphinema* species belonging to taxonomic group II as defined by the polytomous key of Loof and Luc (1990). These taxa also lack an anterior ovary and were used as outgroups for phylogenetic analysis of ITS-1.

ITS-1 region PCR amplification: Generation of ITS-1 region PCR products, PCR conditions, electrophoresis, and DNA purification were as described by Oliveira et al. (2004b).

RFLP analysis: Five microliters of ITS-1 PCR product amplified from all 14 *X. krugi* populations was digested with 5 units of the restriction enzymes Alu I or Hinf I in 20 µl reaction volumes for 3 hr at 37°C. Digested products were separated on a 10% nondenaturing polyacrylamide gel (Kumar et al., 1999), visualized by staining with ethidium bromide, and photographed with a digital system (UVITEC, Cambridge, UK) under UV-light. Restriction sites were also predicted from DNA sequence data using Bioedit (Hall, 1999).

Sequencing: Purified DNA fragments from *X. krugi* populations (PX11, PX26, PX32b, PX37b, PX38c, PX47b, PX50a, PX57, PX59a, PX65b, and PX76a), *X. longicaudatum* (PX41), *X. surinamense* (PX20b), and *X. variegatum* (PX51b) were sequenced directly in both directions with each primer pair, using a Big Dye Terminator cycle sequencing kit (Applied Biosystems, Warrington, UK), as described by Oliveira et al. (2004b).

Fig. 1. Location of samples from which populations of *Xiphinema krugi*, *X. longicaudatum*, *X. surinamense*, and *X. variegatum* were recorded. Brazilian States: Amapá (AP), Mato Grosso (MT), Mato Grosso do Sul (MS), Minas Gerais (MG), Parana (PR), Rio Grande do Sul (RS), Santa Catarina (SC), and São Paulo (SP).

TABLE 1. Populations of *Xiphinema krugi, X. longicaudatum, X. surinamense* and *X. variegatum* used for molecular studies.[a]

Code	Species	Associated plant	City	State	GenBank
PX02	*X. krugi*	Natural vegetation	Pelotas	RS	—
PX11*	*X. krugi*	Natural vegetation	Caconde	SP	DQ017149
PX26*	*X. krugi*	*Vitis* spp.	Garibaldi	RS	DQ017150
PX32b*	*X. krugi*	Cerrado	Luiz Antonio	SP	DQ017151
PX37b*	*X. krugi*	*Eugenia uniflora*	Florianópolis	SC	DQ017157
PX38c*	*X. krugi*	Medicinal plant	Florianópolis	SC	DQ017152
PX47b*	*X. krugi*	*Mangifera indica*	São José do Rio Preto	SP	DQ017148
PX50a*	*X. krugi*	*Manihot esculenta* and *Cucurbita* sp.	Laranjal do Jari	AP	DQ017154
PX51a	*X. krugi*	*M. esculenta* and *Zea mays*	Laranjal do Jari	AP	—
PX57*	*X. krugi*	Natural vegetation	Dourados (Indápolis)	MS	DQ017153
PX59a*	*X. krugi*	Natural vegetation	Dourados	MS	DQ017147
PX65b*	*Xiphinema* sp.	Cerrado	Cuiabá	MT	DQ017155
PX72	*X. krugi*	Natural vegetation	Araxá	MG	—
PX76a*	*X. krugi*	Natural vegetation	São Pedro	SP	DQ017156
PX20b	*X. surinamense*	*Carapa guianensis*	Guarapuava	PR	DQ017146
PX41	*X. longicaudatum*	*Brachiaria decumbens*	Amapá	AP	DQ017145
PX51b	*X. variegatum*	*M. esculenta* and *Z. mays*	Laranjal do Jari	AP	DQ017144

[a] Those populations used for morphological multivariate analysis are marked with an asterisk.

Unfortunately, populations PX02, PX51a, and PX72 consistently yielded poor quality sequence and were excluded from further analysis. Furthermore, populations PX37b and PX76a only yielded approximately 400 bp of high quality sequence at the 3′ end of the ITS-1 region.

Cloning: The presence of visible secondary bands for populations PX11 and PX59a restricted with Hinf I suggested intra-individual variation of the ITS-1 region (Fig. 2a). To investigate the potential intra-individual ITS-1 variation, PCR products from individual nematodes from populations PX11 and PX59a were cloned into a TOPO-TA vector (Invitrogen, Netherlands) using the protocol described by Ritchie et al. (2004). Nine recombinant plasmids (white colonies) were randomly selected, amplified by PCR (PCR conditions as described by Oliveira et al., 2004a), digested with restriction enzyme Alu I, and separated on a 10% nondenaturing polyacrylamide gel (Kumar et al., 1999). The RFLP profiles of the recombinant plasmids were compared to those generated from genomic DNA. A representative plasmid of each of the clone types produced was selected (Fig. 3), purified using a Wizard miniprep (Promega, UK), and sequenced as described above.

Multiple alignment and phylogenetic analysis: Multiple sequence alignments and phylogenetic analyses were done as described by Oliveira et al. (2004a). Pairwise determination of identity between sequences was done

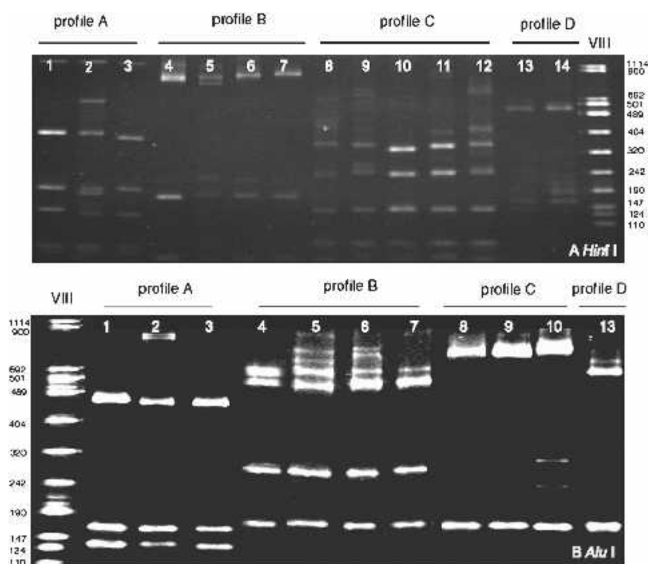

FIG. 2. RFLPs revealed by digestion of ITS-1 region from 14 *X. krugi* populations with two restriction enzymes (A: Hinf I and B: Alu I). Digested products were separated on a 10% nondenaturing polyacrylamide gel. Lanes 1: PX47b; 2: PX59a; 3: PX72; 4: PX02; 5: PX11; 6: PX26; 7: PX32b; 8: PX37b; 9: PX50a; 10: PX57; 11: PX38c; 12: PX76a; 13: PX51a; 14: PX65b. ITS-1 PCR products from populations PX38c; PX76a and PX65b were not available for digestion with Alu I. Bands above 501 bp in lanes 1–3 on Hinf I gel and 2, 4–7 on Alu I gel are undigested products. The fainter bands are partial digest products. VIII = molecular marker VIII (Boehringer).

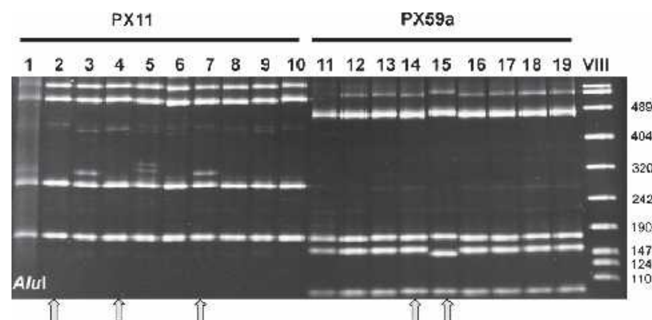

FIG. 3. RFLPs revealed by digestion of ITS-1 region from PCR of genomic DNA and recombinant plasmids derived from an individual of two *X. krugi* populations (PX11 and PX59a) with Alu I. Digested products were separated on a 10% nondenaturing polyacrylamide gel. Lanes 1: PX11 (genomic DNA); 2–10: recombinant plasmids from PX11 (clones 1–9); 11: PX59a (genomic DNA); 12–19: recombinant plasmids from PX59a (clones 1–8). The top bands in each lane are undigested products. Arrows indicate the recombinant plasmids selected for sequencing. VIII = molecular marker VIII (Boehringer).

using MatGAT (Campanella et al., 2003). TREE-PUZZLE (Strimmer and von Haeseler, 1996) was used to estimate the expected transition/transversion rate (Ts/Tv) and alpha shape parameters for a F84 plus gamma + invariant rate heterogeneity model. This model was chosen after a comparison of three models: F84 with uniform rates, F84 plus Gamma, and F84 plus Gamma and a proportion of invariable sites. The statistical comparison of these three nested models was carried out manually using the Likelihood Ratio Test (LRT). Model selection was tested with MODELTEST (Posada and Crandall, 1998), which suggested much simpler nucleotide substitution models, namely K80+G (Fig. 4) and K80 (Fig. 5) than that proposed by TREE-PUZZLE. Assessing optimality between models using a LRT yielded scores of 10.54 (df = 4) and 13.54 (df = 4) compared to significance thresholds (p = 0.05) of 9.49 and 12.59, respectively. Re-analyses of these two datasets did not alter the tree topology (data not shown). The F84 plus gamma + invariant rate hetero-

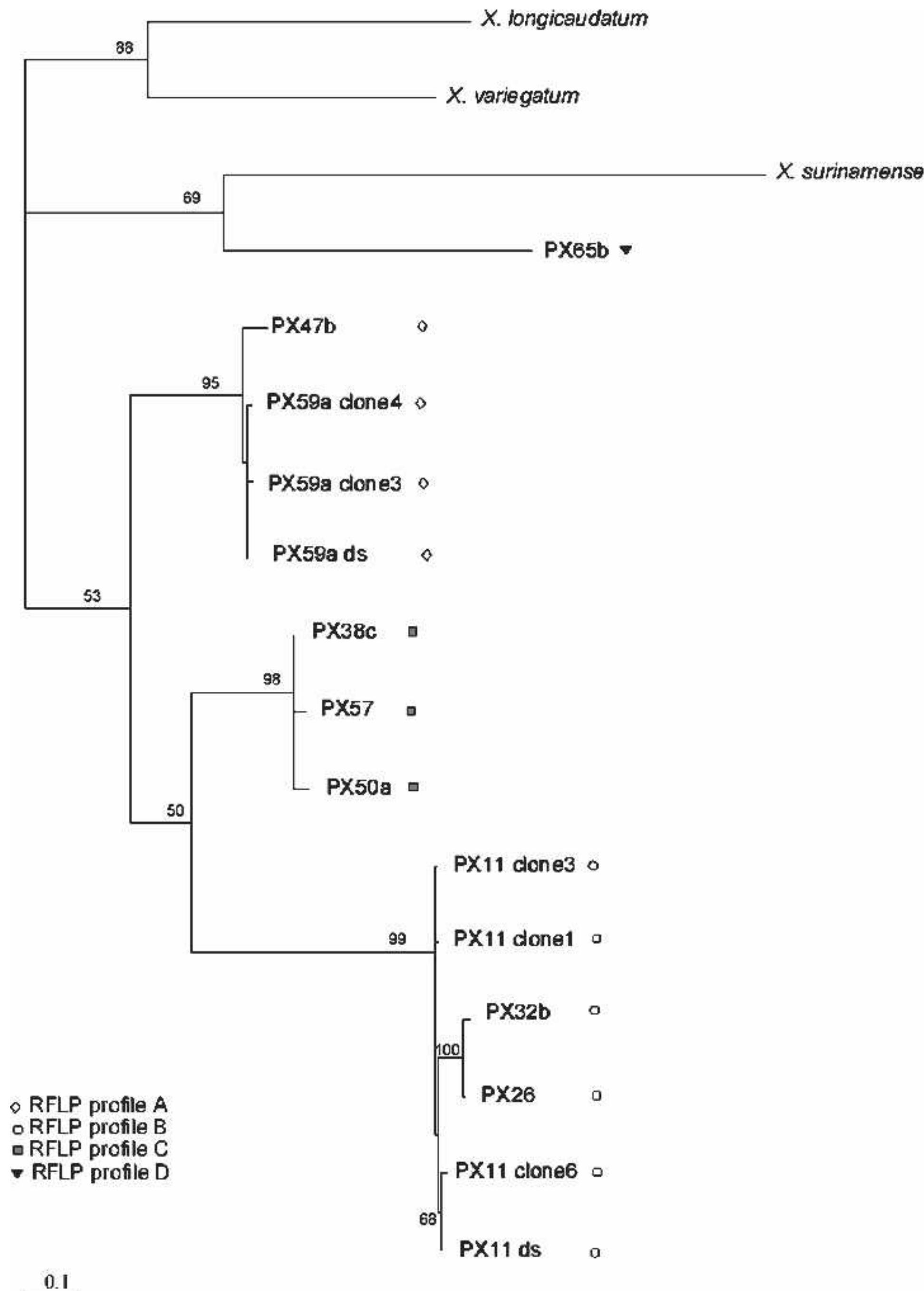

FIG. 4. A Maximum Likelihood tree showing relationships between *X. krugi* populations based on complete ITS-1 sequences. Numbers indicate the bootstrap values >50. Branch lengths are drawn proportional to the number of changes inferred. Direct sequences and clones from populations PX11 and PX59a are indicated by ds and clone, respectively.

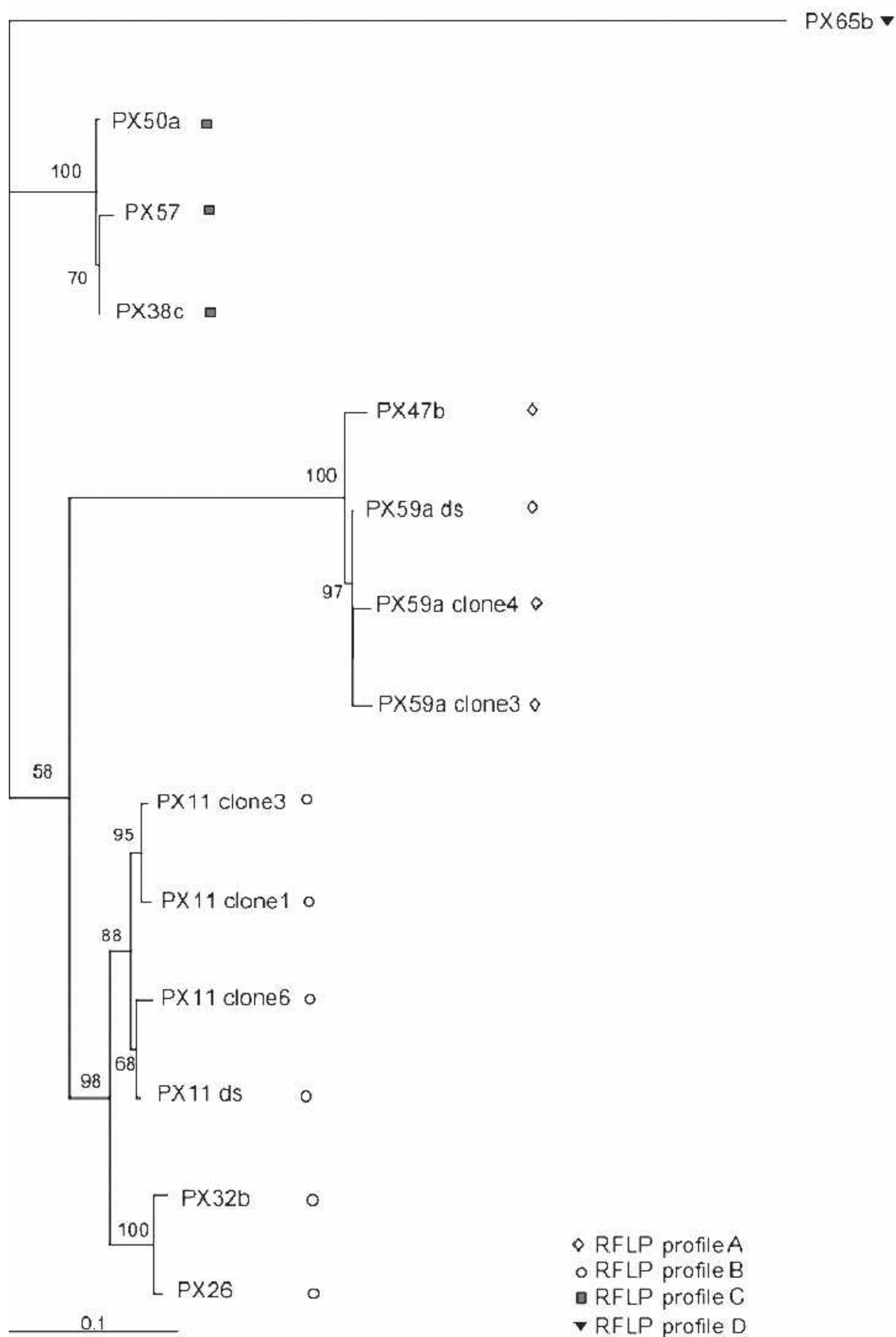

FIG. 5. A Maximum Likelihood tree showing relationships between *X. krugi* populations based on ITS-1 sequences (alignment with selected columns >60% homology). Numbers indicate the bootstrap values >50. Branch lengths are drawn proportional to the number of changes inferred. Direct sequences and clones from populations PX11 and PX59a are indicated by ds and clone, respectively.

geneity model selected for the final analysis (Fig. 6) was deemed most appropriate by MODELTEST.

Phylogenetic analysis was carried out using the Maximum Likelihood (ML) approach. ML was preferred to Maximum Parsimony 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). Maximum Likelihood (ML) trees were estimated (Ts/Tv set at 1.07, alpha at 0.87, 4 gamma rate categories and number of invariable sites = 0 for the complete ITS-1 region alignment; Ts/Tv set at 1.65 and alpha at 2.44 for an alignment with selected columns >60% homology; Ts/Tv set at 1.34 and alpha at 0.84 for

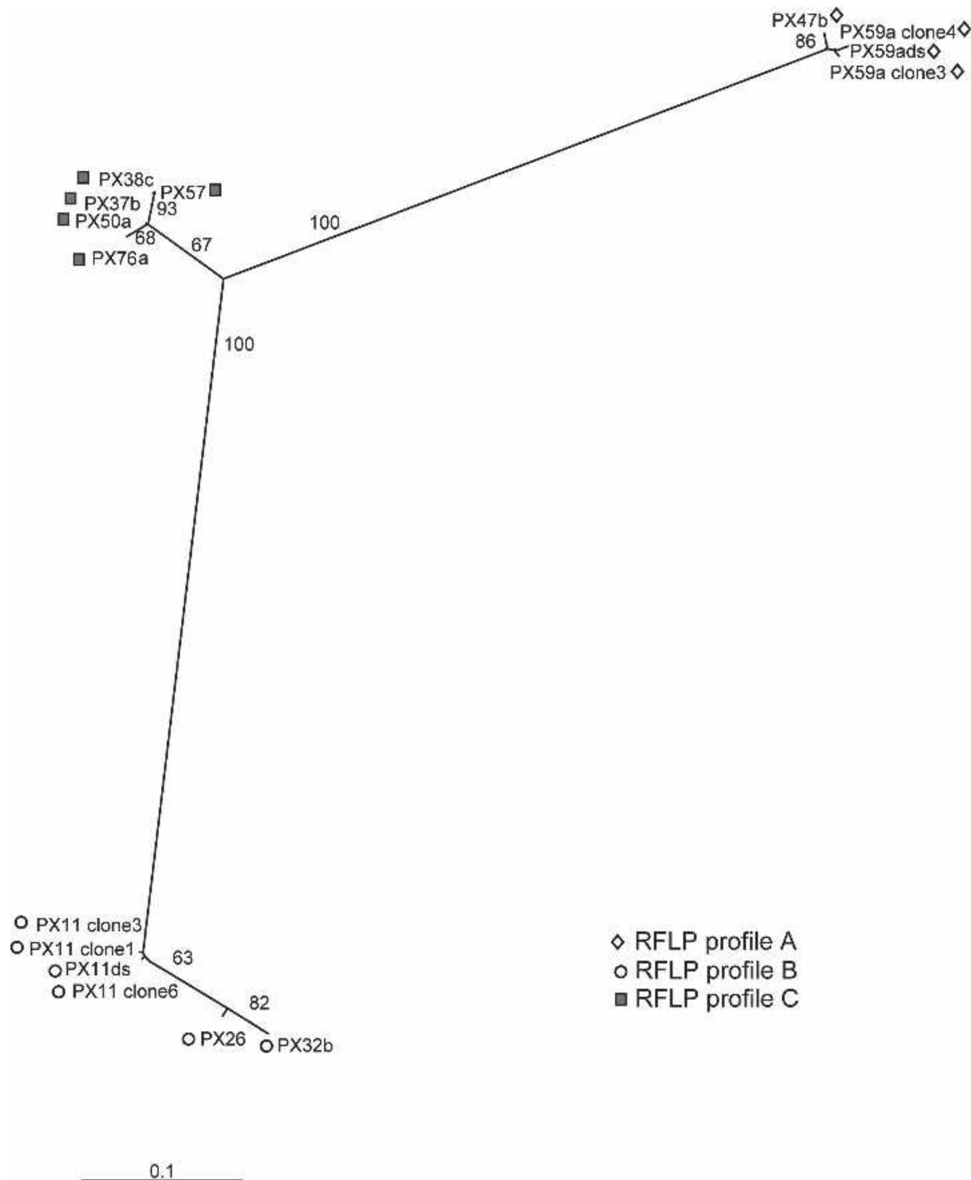

Fig. 6. A Maximum Likelihood radial tree (unrooted) showing relationships between *X. krugi* populations based on 400 bp sequences adjacent to the 3′ end of the ITS-1 region. Numbers indicate the bootstrap values >50. Branch lengths are drawn proportional to the number of changes inferred. Direct sequences and clones from populations PX11 and PX59a are indicated by ds and clone, respectively

alignment of the 3′ end of the ITS-1 region) using the PHYLIP v.3.6a DNAML sub-routine (Felsenstein and Churchill, 1996).

Principal Component Analysis (PCA): To assess the degree of morphometric variability in the *X. krugi* populations studied (Table 2), including the type population (Lordello, 1955), mean values of seven morphometric characters (L, length of both odontostyle and odontophore, tail length, body diameter at anus, maximum body diameter and V) were used in a principal component analysis (PCA).

Results

RFLP analysis: Digestion of ITS-1 PCR products using the restriction enzymes Alu I or Hinf I yielded repeatable patterns that clearly separated 14 different populations into four distinct RFLP profiles (Fig. 2b), confirmed by the expected sizes predicted from ITS-1 sequence data (Table 3). One profile, denoted as profile A, was comprised of populations PX47b, PX59a, and PX72; profile B was comprised of populations PX02, PX11, PX26, and PX32b; profile C was comprised of populations PX37b, PX38c, PX50a, PX57, and PX76a; whilst profile D was represented by populations PX51a and PX65b.

ITS-1 products from individual *X. krugi* females (populations PX11 and PX59a) were cloned into plasmids. Variability was noted between clones from both populations (Fig. 3). Two out of nine cloned PCR products (clones 2 and 6 from population PX11) were represented by a different profile when restricted with Alu

TABLE 2. Morphological and morphometric data of *Xiphinema krugi* populations from Brazil.[a] Values are mean ± standard deviation (range).

Code	PX11	PX26	PX32b	PX37b	PX38c	PX47b	PX50a	PX57	PX59a	PX65b	PX76a	*X. krugi* type[b]
n	5	2	6	5	5	6	4	13	5	4	2	*
L (mm)	2.1 ± 0.1 (2.0–2.2)	2.2, 2.3	1.9 ± 0.1 (1.7–2.1)	1.9 ± 0.1 (1.7–2.0)	2.1 ± 0.1 (2.0–2.2)	1.9 ± 0.1 (1.8–2.0)	1.9 ± 0.1 (1.8–2.0)	2.0 ± 0.1 (1.8–2.1)	1.8 ± 0.1 (1.7–1.9)	1.9 ± 0.1 (1.8–2.0)	1.9, 2.2	(2.1–2.2)
Odontostyle (μm)	120 ± 3.6 (114–124)	115, 123	111 ± 2.4 (107–114)	113 ± 3.3 (109–116)	112 ± 3.3 (107–115)	108 ± 1.7 (105–110)	117 ± 1.0 (116–118)	116 ± 3.7 (108–121)	105 ± 3.7 (100–110)	125 ± 2.6 (122–127)	120, 122	(116–120)
Odontophore (μm)	73 ± 0.9 (73–75)	69, 79	65 ± 1.3 (63–66)	72 ± 1.6 (71–75)	68 ± 1.3 (66–70)	68 ± 1.8 (66–71)	70 ± 1.7 (68–72)	69 ± 3.0 (64–74)	65 ± 0.6 (64–65)	65 ± 1.1 (64–67)	69, 72	(68–72)
Spear (μm)	193 ± 4.1 (187–197)	192, 194	176 ± 2.3 (173–179)	185 ± 4.1 (180–189)	181 ± 3.5 (175–185)	176 ± 2.9 (172–179)	187 ± 2.6 (184–190)	185 ± 6.1 (175–192)	170 ± 4.3 (164–175)	191 ± 2.8 (187–193)	188, 194	(184–192)
Tail length (μm)	29 ± 0.9 (28–30)	31, 31	33 ± 2.6 (28–35)	32 ± 3.5 (28–37)	31 ± 2.4 (28–35)	37 ± 2.2 (34–41)	29 ± 1.0 (27–29)	29 ± 3.9 (24–36)	36 ± 2.8 (32–38)	35 ± 1.7 (34–37)	30, 34	(31–32)
Tail shape[c]	subd	subd	dig	subd	subd	dig	subd	subd	dig	dig	subd	subd
Body diam. (μm)	33 ± 1.8 (31–35)	31, 34	29 ± 1.4 (26–30)	29 ± 1.2 (28–31)	29 ± 1.1 (28–31)	27 ± 1.3 (26–29)	31 ± 1.7 (30–34)	31 ± 2.3 (28–35)	28 ± 0.9 (27–29)	25 ± 1.1 (24–26)	28, 30	*
Maximum body diam. (μm)	45 ± 3.2 (40–48)	42, 46	37 ± 2.5 (34–40)	44 ± 0.7 (43–45)	52 ± 2.8 (48–54)	44 ± 1.4 (43–47)	45 ± 3.7 (41–49)	50 ± 4.3 (43–58)	42 ± 0.5 (41–43)	36 ± 1.7 (34–38)	46, 54	(48–55)
V%	39 ± 0.6 (39–40)	40, 42	41 ± 0.8 (40–42)	33 ± 1.4 (32–36)	33 ± 1.2 (32–34)	32 ± 0.7 (31–32)	34 ± 0.8 (34–35)	39 ± 4.3 (33–44)	34 ± 2.4 (30–36)	38 ± 1.3 (37–40)	32, 34	(33–34)
a	47 ± 2.3 (45–51)	48, 54	51 ± 2.3 (47–53)	43 ± 2.5 (40–46)	41 ± 2.0 (39–44)	43 ± 1.1 (42–44)	43 ± 1.3 (42–45)	40 ± 3.1 (36–47)	43 ± 1.2 (41–44)	53 ± 1.0 (52–54)	41, 41	(38–44)
c	74 ± 4.1 (70–79)	71, 73	58 ± 3.8 (54–63)	60 ± 4.4 (53–64)	69 ± 5.7 (61–74)	52 ± 3.1 (48–57)	68 ± 5.7 (63–74)	70 ± 8.8 (52–85)	51 ± 3.7 (47–55)	54 ± 3.9 (50–58)	56, 74	(66–70)
c′	0.9 ± 0.1 (0.8–1.0)	0.9, 1.0	1.1 ± 0.1 (1.1–1.2)	1.1 ± 0.1 (1.0–1.3)	1.1 ± 0.1 (1.0–1.2)	1.4 ± 0.1 (1.2–1.5)	0.9 ± 0.0 (0.9–1.0)	0.9 ± 0.1 (0.8–1.1)	1.2 ± 0.1 (1.2–1.4)	1.4 ± 0.1 (1.3–1.5)	1.0, 1.2	*

[a] Characters highlighted in bold were used in the principal component analysis.
[b] Range of measurements from Lordello (1955). Asterisk indicates data not provided by the author in the original description.
[c] dig = tail digitate and subd = tail subdigitate.

Table 3. *Xiphinema krugi* population restriction fragment sizes in bp.

Code	Hinf I	Alu I	Profile
PX47b	20 29 50 68 129 171 369	15 135 169 517	A
PX59a ds	20 29 48 68 124 156 369	15 141 169 489	A
PX59a clone3	20 29 49 124 157 445	15 142 169 498	A
PX59a clone4	20 29 48 69 124 164 363	15 135 169 498	A
PX11 ds	56 116 736	169 262 477	B
PX11 clone1	56 116 736	169 262 477	B
PX11 clone3	56 118 732	168 262 477	B
PX11 clone6	56 114 736	169 264 473	B
PX26	58 110 751	169 260 490	B
PX32b	58 112 754	169 268 487	B
PX50a	20 36 85 129 222 322	169 645	C
PX57	20 34 58 73 83 220 333	169 652	C
PX38c	20 34 56 73 81 215 340	169 650	C
PX65b	37 41 121 153 548	169 179 552	D

I. Similarly, one ITS-1 recombinant plasmid (clone 4) from PX59a was dissimilar to the other seven clones analyzed.

Nine complete ITS-1 region sequences derived from populations (PX11, PX26, PX32b, PX38c, PX47b, PX50a, PX57, PX59a, and PX65b) of *X. krugi* that represented each of the four RFLP profiles (Fig. 2) were sequenced directly, and five complete ITS-1 region sequences derived from clones (PX11 clones 1, 3 and 6 and PX59a clones 3 and 4) were also obtained. After excluding nucleotides from both the 18S and 5.8S regions, the length of the trimmed ITS-1 sequences ranged from 649 bp (PX59a) to 759 bp (PX32b). A multiple sequence alignment, including the outgroups *X. longicaudatum, X. variegatum,* and *X. surinamense,* yielded a consensus length of 926 bp, of which only 92 (9.9% of all sites) were constant. The estimated average nucleotide frequencies were: 25.8% (T), 26.7% (A), 25.0% (G), and 22.5% (C).

Divergence between the four RFLP profiles (including clones) was greater than that for populations within each profile. The genetic distance between the four RFLP profiles was as great as that between the profiles and putative *Xiphinema* species that also lacked an anterior ovary, e.g., *X. longicaudatum, X. surinamense,* and *X. variegatum* (data not shown; range 0.632: PX59a ds and *X. variegatum* to 1.425: PX65b and *X. surinamense*). Furthermore, sequence divergence of the clones ranged from 1.1% (PX11 clones 1 vs. 3) to 2.4% (PX11 clones 1 and 3 vs. 6), corresponding to 11 and 30 substitutions, respectively.

Phylogenetic analyses: A ML consensus tree (Fig. 4) separated populations of *X. krugi* that were sequenced into four mostly well resolved groups in direct agreement with the RFLP profiles. From the ML tree, it was evident that population PX65b was not *X. krugi,* thus a new alignment was generated replacing *X. longicaudatum, X. surinamense,* and *X. variegatum* with PX65b as the outgroup. Following Oliveira et al. (2004a), sequences with >60% homology (http://www.treebase.org submission ID number S1655) were identified using GeneDoc (Nicholas et al., 1997), and ambiguous regions were excluded from a new phylogenetic analysis. A new alignment yielded a consensus length of 986 bp, of which 212 (21.5% of all sites) were constant. Estimated average nucleotide frequencies among the populations were: 26.0% (T), 27.2% (A), 24.7% (G), and 22.0% (C). As with the previous ML tree (Fig. 4), this new ML analysis (Fig. 5) yielded four groups (including PX65b) that matched the initial RFLP profiles.

Three populations (PX37b, PX51a, and PX76a) consistently proved problematical to sequence directly. However, using only the reverse primer, partial ITS-1 sequences of approximately 400 bp adjacent to the 3′ end were obtained for populations PX37b and PX76a, but unfortunately not PX51a. Based on this 400 bp segment, a new overall alignment was generated which was further improved by excluding the outgroup (PX65b). This alignment (http://www.treebase.org submission ID number S1655) yielded a consensus length of 465 bp, of which 135 (29% of all sites) were constant. Estimated average nucleotide frequencies among the populations were: 27.4% (T), 29.3% (A), 22.8% (G), and 20.4% (C). As before, the ML consensus tree (Fig. 6) clearly separated the *X. krugi* populations into groups concurring with the RFLP profiles (note profile D represented by PX65b was excluded).

Multivariate statistical analysis: Of the 14 *X. krugi* populations in this study, only 11 populations plus morphometric data from the type population (Lordello, 1955) (Table 2) were used in a principal component analysis of eight morphometric characters. Populations PX02, PX51a, and PX72 were excluded due to the lack of corresponding ITS-1 sequence data.

The PCA separated the 12 *X. krugi* populations (Table 2) into four distinct clusters (Fig. 7), the composition of which, at the population level, corresponded directly to that produced by both RFLP and phylogenetic analyses (Figs. 2,4,5,6). The only exception was population PX32b which was morphometrically closer to population PX65b (RFLP profile D) but genetically similar to RFLP profile B (population PX11 and PX26).

A morphometric examination of PX65b revealed that specimens from this population differed from the *X. krugi* type (Lordello, 1955) by having a longer odontostyle (125 vs. 116–120 µm), tail (35 vs. 32 µm) and a more posterior vulva location (38 vs. 34%). Thus, it is unlikely that PX65b is *X. krugi* sensu stricto, as supported by both molecular and morphometric data.

Discussion

PCR-RFLP and sequencing of ITS-1 region rDNA have previously been used to assess the genomic variability amongst populations of plant-parasitic nematodes including: *Belonolaimus longicaudatus* (Cherry et al., 1997), *Globodera pallida* (Blok et al., 1998; Grenier et

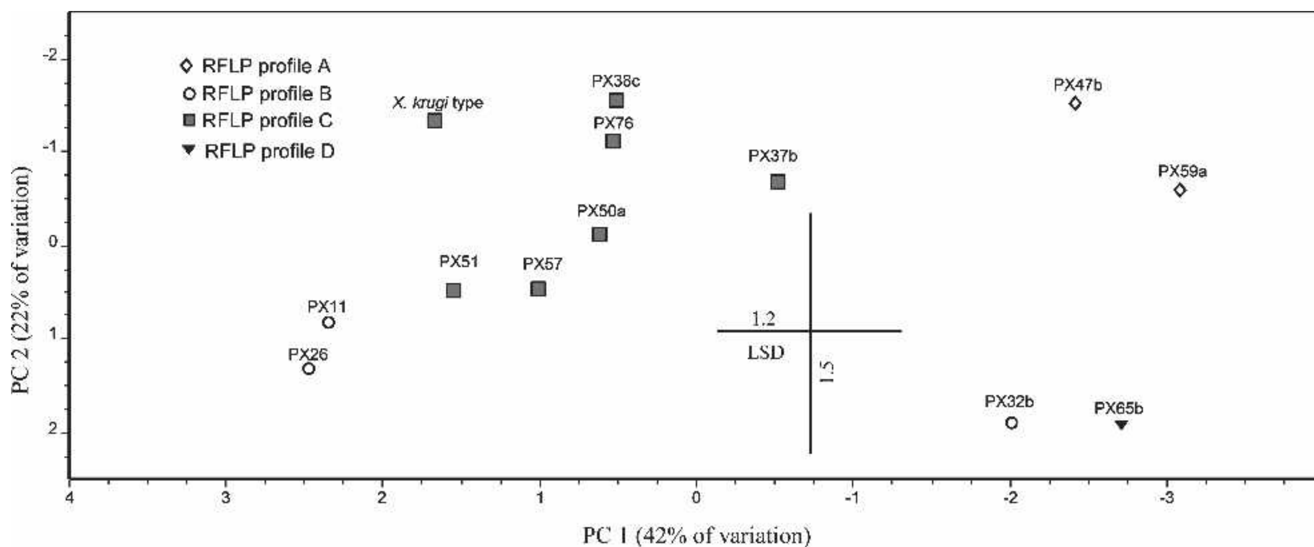

FIG. 7. Principal component analysis of 12 *X. krugi* populations (Table 2) based on mean values of seven morphometric characters.

al., 2001), *Heterodera glycines* and *H. avenae* (Zheng et al., 2000), *Nacobbus aberrans* (Reid et al., 2003), *Radopholus similis* (Elbadri et al., 2002), *Xiphinema americanum*-group (Vrain et al., 1992), *X. elongatum* (Chen et al., 2004b), *X. hunaniense* (Chen et al., 2004a), and *X. insigne* (Chen et al., 2004c).

Here, a RFLP analysis using Alu I and Hinf I clearly separated 14 morphologically putative *X. krugi* populations into four different profiles that were confirmed by independent sequencing of the ITS-1 region and subsequent ML phylogenetic analyses. This was further supported by a principal component analysis of morphometric characters that yielded four "clusters". The only exception was population PX32b that clustered with population PX65b from a different molecular profile. The reason for this is unclear; however, intra-population variability could have been present but undetected due to the limited number of specimens available for analysis. Furthermore, it is possible that intra-individual variation occurred in the ITS-1 region but only one type was amplified when sequenced directly. This merits further investigation.

ITS-1 sequence homology was greater amongst populations (92.6–97.4%) that represented the same RFLP profile than between profiles (62.2–78.7%). The degree of homology was similar to intraspecific variability reported for other longidorid populations, e.g., *Longidorus biformis* (89.1–99.4%; Ye et al., 2004) and *Xiphidorus minor* (95%; Oliveira et al., 2004a). The observed homology between sequences that represented RFLP profiles A to D was also similar to that found between putative but morphologically closely related *Xiphinema* species. For example, RFLP profiles A and B had 68.0% ITS-1 sequence homology, which was comparable to that displayed between profile A and *X. variegatum* (66.1–64.6%).

Notwithstanding the variability of *X. krugi* at a population level, intra-individual variability was also noted. The sequences of recombinant plasmids from individuals of populations PX11 and PX59a differed by up to 2.4% within a population. Although levels of intra-individual variability can be sufficiently large to provide problems interpreting phylogenetic analysis (Leo and Barker, 2002), intra-individual variability in this study was insufficient to separate the recombinant plasmids from directly sequenced DNA of the same population in the phylogenetic analysis.

The application of molecular methods to studies of nematode population structure and systematics has revealed that some long assumed single species are in fact cryptic species that are genetically distinct but share morphological similarities. Cryptic species of brackish-water (Moens and Vincx, 2000) and animal (Chilton et al., 1992; Bernardi and Goswami, 1997; Hoberg et al., 1999; Hung et al., 1999; Gaffney, 2000; Zhu et al., 2000), and plant-parasitic nematodes (De Ley et al., 1999; Ye et al., 2004) have been reported. Our results provide some evidence that *X. krugi* is a species complex comprised of at least four distinct genotypes, some of which may be cryptic species. Future studies on *X. krugi*, such as mtDNA prospecting (Blouin et al., 1998; Blouin, 2002) may provide more conclusive evidence.

The present study clearly demonstrates potential problems for nematode taxonomy. In light of an ever-decreasing classical taxonomy infrastructure (Andre et al., 2001; Coomans, 2002), the future of nematode taxonomy in a molecular era will be challenging. Such challenges may be further compounded by regional variability in the levels of cryptic speciation. Herbert et al. (2004) noted that cryptic species are prevalent in tropical regions and suggested a broad-ranging assessment of their incidence to determine levels of hidden diversity. For 50 years, *X. krugi* was assumed by many classical taxonomists to be a morphologically variable

species (Frederick and Tarjan, 1974; Lamberti and Tarjan, 1974; Luc and Hunt, 1978; Ferraz, 1980). However, this study, encapsulating both molecular and classical taxonomic data, has demonstrated the possibility that in fact *X. krugi* is a species complex comprised of four distinct genotypes and/or cryptic species that have a morphological basis, albeit defined by minor morphometric differences.

At some point, the delineation of a "species" based solely on sequence data will have to be addressed. Such decisions in the future will have to consider the impact of intra- and inter-population variability. Although recognized in classical taxonomy, few if any molecular studies have dealt with this issue. Paradoxically, Lee (2004) asserted that the application of such molecular techniques can be useful in species taxonomy, including nematodes, only when combined with taxonomic skills which as already noted are depleted. However, it is imperative that studies that aim to make definitive statements about Nematoda at the species level, e.g., NemaTol and diagnostics, address natural variation.

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