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Molecular characterization and diagnostics of stubby root and virus vector nematodes of the family Trichodoridae (Nematoda: Triplonchida) using ribosomal RNA genes

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Plant parasitic nematodes of the family Trichodoridae cause substantial yield losses in many agricultural crops. Rapid and accurate identification of trichodorids to the species level is critical for selection of appropriate measures for control. This study analysed 99 sequences of the D2–D3 expansion segments of the 28S rRNA gene and 131 sequences of the 18S rRNA gene from the stubby nematodes belonging to the genera *Nanidorus*, *Paratrichodorus* and *Trichodorus*. Species delimiting was based on the integration of morphological identification, which is not provided in the present article, and molecular-based phylogenetic inference and sequence analysis. Twenty-two valid species and several species complexes were identified among nematodes included in the analysis. PCR-RFLPs of the partial 18S rDNA and the D2–D3 expansion segments of the 28S rDNA were tested and proposed for identification of these nematodes. Gel PCR-RFLP profiles and tables with restriction fragment lengths for several diagnostic enzymes are provided for identification. Some problems of taxonomy and phylogeny of nematodes of the family Trichodoridae are also discussed.

Keywords: 18S rDNA, D2–D3 of 28S rDNA, *Nanidorus*, *Paratrichodorus*, PCR-RFLP, *Trichodorus*

Introduction

Nematodes of the family Trichodoridae are widely distributed in Europe and North America and are also reported from other parts of the world. These nematodes can cause substantial crop losses by acting as plant pathogens and as vectors for plant viruses. Because trichodorid nematode feeding can cause stunting of the roots, they are referred to as stubby root nematodes. One hundred and two species belonging to five genera, *Allotrichodorus*, *Monotrichodorus*, *Nanidorus*, *Paratrichodorus* and *Trichodorus* are currently recognized in the Trichodoridae family. Several stubby nematode species are known to vector tobraviruses, which cause economically important diseases in several crops (Decraemer, 1995; Decraemer & Robbins, 2007). Viruses belonging to the genus *Tobravirus* include *Tobacco rattle virus*, *Pea early-browning virus* and *Pepper ringspot virus*. There is a highly specific relationship between virus and nematode vector, so that particular virus isolates are transmitted only by certain nematode species.

Rapid and accurate identification of trichodorids to the species level is the first critical step for selection of appropriate measures for control of these nematodes. Traditional identification of trichodorids is based on analysis of morphological and morphometrical characters, which often have high intraspecific variability, are complex, difficult and time-consuming. Recently, DNA-based approaches have been successfully adapted for the molecular diagnostics of trichodorids. Blaxter *et al.* (1998), Boutsika *et al.* (2004b), Riga *et al.* (2007), Van Megen *et al.* (2009) and Duarte *et al.* (2010) have published sequences of 18S rRNA and ITS rRNA genes for several agriculturally important species of trichodorids. Using sequence data, Boutsika *et al.* (2004a) and Riga *et al.* (2007) developed a PCR with specific primers for diagnostics of *P. allius*, *P. macrostylus*, *P. pachydermus*, *P. teres*, *T. primitivus* and *T. similis*. Holeva *et al.* (2006) designed a real-time PCR assay for detection and quantification of *P. pachydermus* and *T. similis* in field samples. Recently, Duarte *et al.* (2011) developed a PCR-RFLP assay based on the 18S rRNA gene for rapid identification of 12 trichodorid species belonging to the genera *Nanidorus*, *Paratrichodorus* and *Trichodorus*. DNA techniques have been successfully applied to diagnostics of several stubby nematode species. However, many species remain uncharacterized at the molecular level.

The main objectives of this study were to: (i) verify species identification of trichodorid nematodes collected in the Czech Republic, USA and India by using phylogenetic analysis of rRNA gene sequences; (ii) develop a

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PCR-RFLP assay using D2–D3 expansion fragments of the 28S rRNA gene for diagnostics of stubby nematodes; and (iii) test the PCR-RFLP assay developed by Duarte *et al.* (2011) with a wider range of trichodorid samples.

Materials and methods

Nematode populations

Nematode populations used in this study were obtained from soil samples collected from the Czech Republic, India and USA (California) (Table 1). The nematodes from the USA were extracted from samples using a centrifugal flotation technique (Coolen, 1979; Hooper, 1986a) and the nematodes from the Czech Republic and India were extracted by a sieving and decanting method (Brown & Boag, 1988). Specimens were killed by gentle heat, fixed in 4% formalin or triethanolamine–formalin (TAF, 2% triethanolamine, 7% formaldehyde solution, 91% water) and mounted in anhydrous glycerin for examination (Hooper, 1986b). Morphological identification of specimens was done using keys provided by Decraemer (1995) and Decraemer & Baujard (1998), with corresponding species descriptions. Some of the species from the Czech Republic used here were described morphologically by Kumari (2010) and Kumari & Decraemer (2011). In this study, the species were defined and delimited based on an integrated approach that considered morphological evaluation, molecular based phylogenetic inference (tree based methods) and sequence analysis (genetic distance methods) (Sites & Marshall, 2004).

DNA isolation, amplification, cloning and sequencing

Molecular studies of trichodorid samples from the Czech Republic, India and California, USA were conducted using slightly different protocols at the Crop Research Institute (Czech Republic) and at the California Department of Food and Agriculture (USA). Trichodorid nematodes collected from the Czech Republic and India were stored in 1 M NaCl before analysis. Total genomic DNA from individual nematodes was extracted according to the rapid method of Stanton *et al.* (1998). Four regions, 18S, ITS1, ITS2 and partial 28S of rRNA genes, were amplified using nematode universal primers (Table 2). The 18S rRNA gene was amplified into three fragments. Primer combinations were as follows: first fragment SSU_F_04 + SSU_R_09, second fragment SSU_F_22 + SSU_R_13, and third fragment SSU_F_23 + SSU_R_81. PCR was performed in a 25 μL total volume containing one PCR bead (GE Healthcare), 20·5 μL double distilled sterile water and 2·0 μL each primer (10 pmol μL^{-1}) (synthesized by Generi Biotech). To this, 0·5 μL of DNA was added as a template for PCR. A negative control (sterilized water) was included in all PCR experiments. All PCR reactions were performed on a DNA Engine PTC–1148 thermal cycler (Bio-Rad). The cycling profile for all four markers was as follows: initial denaturation for 3 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, with a final extension at 72°C for 10 min. Amplicons were analysed by electrophoresis and the remaining products were purified using the High Pure Product Purification kit (Roche Diagnostics GmbH) and sequenced in both directions using each primer pair (Macrogen). SEQUENCHER 4.8 (Gene codes Corp.) was used to assemble and view each sequence and check for base-calling errors.

For stubby nematode species collected in the USA, DNA was extracted using the proteinase K protocol. Several specimens from each sample were put into a drop of water on a glass slide and cut under a binocular microscope. Each nematode specimen was transferred to an Eppendorf tube containing 25 μL double distilled water, 2 μL 10 × PCR buffer and 3 μL proteinase K (600 μg mL^{-1}) (Promega). Tubes were incubated at 65°C for 1 h and then at 95°C for 15 min. Detailed protocols for PCR, cloning and sequencing were as described by Tanha Maafi *et al.* (2003). The PCR product was purified using the QIAquick Gel Extraction Kit (QIAGEN). The primer sets used for amplification and sequencing of ribosomal RNA gene fragments are given in Table 2. PCR products were purified and run on a DNA multicapillary sequencer at the University of California, Riverside.

Sequences were submitted to GenBank under accession numbers as indicated in Table 1.

PCR-RFLPs

The PCR product of the 18S rRNA gene was digested with *Taq*I, *Sat*I, *Bse*NI or *TscA*I. The PCR product of D2–D3 expansion fragments of the 28S rRNA gene was digested with *Bse*NI, *Pst*I, *Pvu*II or *Rsa*I. Three to five microlitres of purified PCR products were digested with each of the restriction enzymes. RFLPs were separated by electrophoresis using TAE-buffered gels, stained with ethidium bromide, visualized using a UV transilluminator and photographed. The length of each restriction fragment was obtained by virtual digestion of each sequence using WEBCUTTER 2.0 (http://www.firstmarket.com/cutter/cut2.html).

Sequence and phylogenetic analysis

The new sequences of the 18S rRNA gene and D2–D3 expansion fragments of 28S rRNA gene were aligned using CLUSTALX 1.83 (Thompson *et al.*, 1997) using default parameters of corresponding gene sequences (Blaxter *et al.*, 1998; Boutsika *et al.*, 2004a; Van Megen *et al.*, 2009; Duarte *et al.*, 2010; RC Holeva, MS Phillips, FG Wright, DJ Brown and VC Blok, the James Hutton Institute, Dundee, UK, unpublished data, XQ Li and JW Zheng, Institute of Biotechnology, Zhejiang University, China, unpublished data). Outgroup representatives of the genera *Tripyla* and *Alaimus* for the D2–D3 and *Tobrilus, Prismatolaimus*, *Tripyla, Tylolaimophorus* and *Diphtherophora* for 18S data sets were chosen using previous published data (Van Megen *et al.*, 2009). Sequence data sets were analysed with Bayesian inference

Table 1 Species and populations of trichodorids and outgroup taxon sequenced in the present study

Identification based on morphology and rRNA sequences	Locality	Host plant	Collection code	rRNA genes[a]				Source of materials
				18S	ITS1	ITS2	D2–D3 of 28S	
Nanidorus minor	Lodi, San Joaquin County, CA, USA	Alfalfa	CD465	JN123364	–	JN123380	JN123393, JN123394	Subbotin S.A.
N. minor	Kerman, Fresno County, CA, USA	Cotton	CD474	JN123365	–	JN123381	JN123395, JN123396	Subbotin S.A.
N. renifer	Goulds, FL, USA	Bromeliad	CD541	JN123366	–	–	JN123397, JN123398	Subbotin S.A.
Paratrichodorus pachydermus 'B'	Dobříš, Czech Republic	Beech	XP1	–	–	–	JN123399	Kumari S.
P. pachydermus 'B'	Jeseň, Czech Republic	Maple	PP1	JN123367	JN123376	JN123382	JN123400	Kumari S.
P. pachydermus 'B'	Lhenice, Czech Republic	Sweet cherry	XP2	–	–	–	JN123401	Kumari S.
P. pachydermus 'B'	Maroltov, Czech Republic	Maple	XP3	–	–	–	JN123402	Kumari S.
P. pachydermus 'B'	Obory, Czech Republic	Oak	XP4	–	–	–	JN123403	Kumari S.
P. pachydermus 'B'	Rájov, Czech Republic	Unkown	XP5	–	–	–	JN123404	Kumari S.
P. porosus 'C'	Incline, Mariposa County, CA, USA	Trees	CD606	JN123368	–	JN123383	JN123405, JN123406	Subbotin S.A.
Trichodorus pakistanensis 'A'	Rajot, HP, India	Sacred fig	TINP	JN123369	NA	JN123384	JN123407	Kumari S.
T. primitivus	Ratiškovice, Czech Republic	Peach	TP2	JN123370	NA	JN123385	JN123408	Kumari S.
T. similis	Červeny vrch, Czech Republic	Oak	TS3	JN123371	JN123377	JN123386	DQ832183[b]	Kumari S.
T. sparsus 'A'	Borek, Czech Republic	Grass	XT6	–	–	–	JN123409	Kumari S.
T. sparsus 'A'	Brodce, Czech Republic	Beech	XT7	–	–	–	JN123410	Kumari S.
T. sparsus 'A'	Hlínoviště, Czech Republic	Beech	XT8	–	–	–	JN123411	Kumari S.
T. sparsus 'A'	Chloumek, Czech Republic	Oak	XT9	–	–	–	JN123412	Kumari S.
T. sparsus 'A'	Janov, Czech Republic	Beech	XT10	–	–	–	JN123413	Kumari S.
T. sparsus 'A'	Jetřichovice, Czech Republic	Grass	XT11	–	–	–	JN123414	Kumari S.
T. sparsus 'A'	Kámen, Czech Republic	Beech	XT12	–	–	–	JN123415	Kumari S.
T. sparsus 'A'	Kozí Hory, Czech Republic	Beech	XT13	–	–	–	JN123416	Kumari S.
T. sparsus 'A'	Lysá nad Labem, Czech Republic	Unkown	XT14	–	–	–	JN123417	Kumari S.
T. sparsus 'A'	Mcely, Czech Republic	Unkown	XT15	–	–	–	JN123418	Kumari S.
T. sparsus 'A'	Mezní Louka, Czech Republic	Beech	XT16	–	–	–	JN123419	Kumari S.
T. sparsus 'A'	Obrubce, Czech Republic	Pine	TS4	JN123372	JN123378	JN123387	JN123420	Kumari S.
T. sparsus 'A'	Ohaveč, Czech Republic	Oak	XT17	–	–	–	JN123421	Kumari S.
T. sparsus 'A'	Staré splavy, Czech Republic	Birch	XT18	–	–	–	JN123422	Kumari S.
T. sparsus 'A'	Vojtěchov, Czech Republic	Beech	XT19	–	–	–	JN123423	Kumari S.
T. sparsus 'B'	Borek, Czech Republic	Grass	TS5	JN123373	JN123379	JN123388	JN123424	Kumari S.
T. sparsus 'B'	Přelovice, Czech Republic	Strawberry	XT20	–	–	–	JN123425	Kumari S.
T. variopapillatus	Křivoklát, Czech Republic	Elm	TV6	GQ148719[b]	NA	JN123389	GQ148718[c]	Kumari S.
T. viruliferus	Bílé podolí, Czech Republic	Apple	XT21	–	–	–	JN123426	Kumari S.
T. viruliferus	Ratiškovice, Czech Republic	Peach	TV7	–	NA	JN123390	JN123427	Kumari S.
T. viruliferus	Výsoká, Czech Republic	Maple	TV8	JN123374	NA	JN123391	JN123428	Kumari S.
Trichodorus sp. C	CA, USA	Unknown	CA103	JN123375	–	JN123392	JN123429	Subbotin S.A.
Trichodorus sp. D	Kingsburg, Kings County, CA, USA	Peach	CD488	–	–	–	JN123430, JN123431	Subbotin S.A.
Alaimus sp.	Bílé Podolí, Czech Republic	Apple	FLN	–	–	–	JN123432	Kumari S.

[a]–, not sequenced; NA not acquired.
[b]After Kumari *et al.* (2007).
[c]After Kumari & Decraemer (2009).

Table 2 Primer combinations used in the present study

Gene	Primer code	Direction	Primer sequence 5′–3′	Reference
18S rRNA	SSU_F_04	Forward	GCT TGT CTC AAA GAT TAA GCC	Blaxter *et al.* (1998)
18S rRNA	SSU_R_09	Reverse	AGC TGG AAT TAC CGC GGC TG	Blaxter *et al.* (1998)
18S rRNA	SSU_F_22	Forward	TCC AAG GAA GGC AGC AGG C	Blaxter *et al.* (1998)
18S rRNA	SSU_R_13	Reverse	GGG CAT CAC AGA CCT GTT A	Blaxter *et al.* (1998)
18S rRNA	SSU_F_23	Forward	ATT CCG ATA ACG AGC GAG A	Blaxter *et al.* (1998)
18S rRNA	SSU_R_81	Reverse	TGA TCC WKC YGC AGG TTC AC	Blaxter *et al.* (1998)
18S rRNA	1091-F	Forward	AGG AAT TGA CGG AAG GGC AC	Duarte *et al.* (2010)
18S rRNA	1671-R	Reverse	TCC TCT AAG TAA ATC CCA TTG G	Duarte *et al.* (2010)
ITS1 rRNA	BL18	Forward	CCC GTC GMT ACT ACC GAT T	Boutsika *et al.* (2004a)
ITS1 rRNA	5818	Reverse	ACG ARC CGA GTG ATC CAC	Boutsika *et al.* (2004a)
ITS2 rRNA	ITSA	Forward	ATC GAT GAA GAA CGC AGC	Boutsika *et al.* (2004a)
ITS2 rRNA	PXb481	Reverse	TTT CAC TCG CCG TTA CTA AGG	Vrain *et al.* (1992)
28S rRNA	D2A	Forward	ACA AGT ACC GTG AGG GAA AGT TG	Nunn (1992)
28S rRNA	D3B	Reverse	TCG GAA GGA ACC AGC TAC TA	Nunn (1992)

(BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). BI analysis under the GTR + I + G model for each gene was initiated with a random starting tree and was run with four chains for 1×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log-likelihood values of the sample points stabilized after approximately 1000 generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given for appropriate clades. Sequence differences between samples were calculated with paup* 4b10 (Swofford, 2003) as an absolute distance matrix and the percentage was adjusted for missing data.

Results

Species identification and delimiting

Ninety-nine and 131 sequences from trichodorids were included in the analyses of the D2–D3 of 28S and 18S rRNA genes, respectively. Forty sequences of the 28S and 12 sequences of the 18S rRNA gene were obtained in the present study. Using traditional morphological characters and molecular criteria (apomorphies and DNA distances), the following 10 species were distinguished within the samples: *Nanidorus minor, N. renifer, Paratrichodorus pachydermus, P. porosus, Trichodorus pakistanensis, T. primitivus, T. similis, T. sparsus, T. variopapillatus* and *T. viruliferus*. Two representatives of *Trichodorus* (*Trichodorus* sp. C and *Trichodorus* sp. D) from California were not identified to a species level. Several samples identified as representative of the same morpho-species showed differences in molecular characteristics, and were thus classified here as different species types: *Trichodorus sparsus* type 'A', 'B', 'C', 'D'; *T. pakistanensis* 'A', 'B'; *P. porosus* 'A', 'B', 'C'; *P. pachydermus* 'A', 'B'; *P. teres* 'A', 'B', 'C'; *P. hispanus* 'A', 'B'. The analysis of the D2–D3 of the 28S rDNA sequence data set revealed four unidentified species of *Trichodorus,* and the partial 18S rDNA sequence data set distinguished four unidentified species of *Trichodorus* and two unidentified species of *Paratrichodorus*. Morphological descriptions and identifications of these nematodes are not available. More detailed morphological and molecular analysis is required to further evaluate and identify these samples. A total of 22 valid known species (*Trichodorus* – 11 species; *Nanidorus* – three species; *Paratrichodorus* – eight species) were identified and included in the analyses.

Sequence and phylogenetic analysis

18S rDNA

The alignment for the partial 18S rDNA included 136 sequences and was 1137 bases long. Fourteen *Trichodorus*, three *Nanidorus* and 10 *Paratrichodorus* nominal and putative species were included in the analysis.

Intraspecific sequence variations for some species were: *P. porosus* 'C', 0–0·5% (0–6 nt); *N. minor,* 0–0·6% (0–7 nt); *N. renifer*, 0–0·4% (0–4 nt); *T. primitivus,* 0–0·3% (0–3 nt); *T. pakistanensis*, 0–0·7% (0–8 nt); *T. sparsus* 'A', 0·2 (2 nt); *T. sparsus* 'B', 0–0·3% (0–4 nt); *T. similis*, 0–0·5% (0–5 nt); and *T. nanjingensis*, 0–0·9% (0–11 nt). The 18S BI tree included a major weakly supported clade with all *Trichodorus* species, one highly supported clade with most *Paratrichodorus* samples, a weakly supported clade with *P. porosus* 'A' and 'B', and two distinct clades with *Nanidorus renifer* and *N. nanus*, respectively, and a group of *N. minor* sequences (Fig. 1). The genus *Trichodorus* was monophyletic, whereas *Paratrichodorus* and *Nanidorus* were shown to be paraphyletic. Relationships between the major clades were not well resolved. *Trichodorus sparsus* 'A', 'B', 'C', 'D', *P. teres* 'A', 'B', 'C' and *P. hispanus* 'A', 'B' formed corresponding groups of related clades, whereas there were no sister relationships for *T. pakistanensis* 'A' with 'B' and *P. porosus* 'C' with 'A'+'B', respectively.

D2–D3 of 28S rDNA

The alignment for the D2–D3 of the 28S rDNA included 102 sequences and was 819 nucleotides in length. Thirteen *Trichodorus*, two *Nanidorus* and three *Paratrichod-*

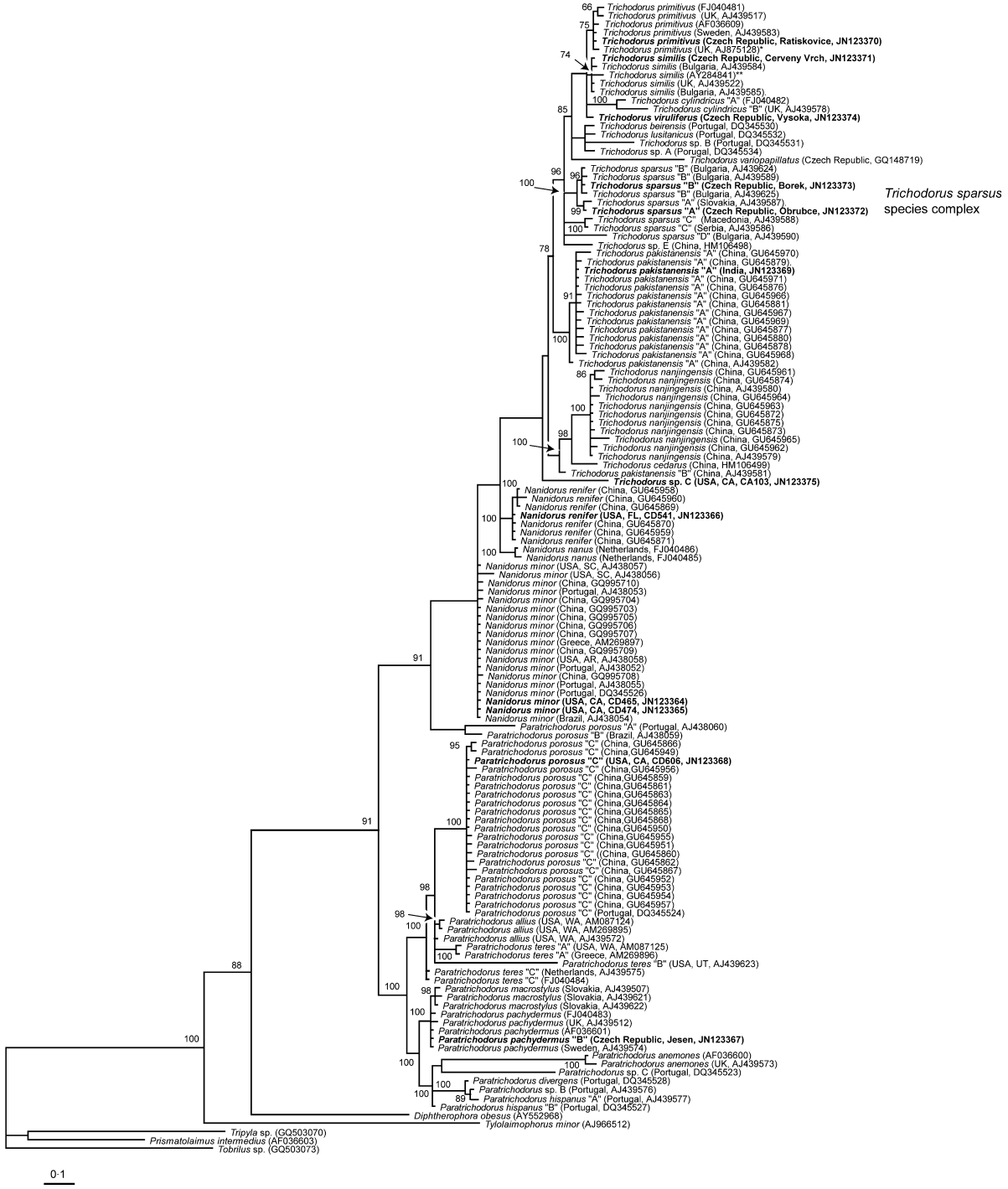

Figure 1 Phylogenetic relationships within trichodorid nematodes: Bayesian 50% majority rule consensus tree from two runs as inferred from partial 18S rRNA gene sequence alignments under the GTR + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are indicated by bold letters. *Originally identified as 'uncultured nematode'; **Originally identified as *Trichodorus variopapillatus*.

orus nominal and putative species were included in this analysis.

Intraspecific sequence variations for some species were: *P. porosus* 'C', 0–0·6% (0–5 nt); *P. pachydermus* 'B', 0% (0 nt); *T. sparsus* 'A', 0% (0 nt); *T. pakistanensis* 'A', 0–0·6% (0–5 nt); *T. primitivus*, 0·3% (2 nt); *N. renifer*, 0–1·6% (0–10 nt); *N. minor*, 0–1·6% (0–11 nt). In the D2–D3 BI tree *Trichodorus* samples were distributed among six moderate or highly supported major clades, and *Nanidorus* and *Paratrichodorus* represented two and

Figure 2 Phylogenetic relationships within trichodorid nematodes: Bayesian 50% majority rule consensus tree from two runs as inferred from D2 to D3 of 28S rRNA gene sequence alignments under the GTR + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are indicated by bold letters. *Only D3 of 28S rRNA gene sequence used.

three clades, respectively (Fig. 2). The genus *Trichodorus* was paraphyletic, whereas *Paratrichodorus* and *Nanidorus* were monophyletic.

ITS1 of rDNA

The ITS1 sequences were obtained from four species. Comparison of the sequences revealed the following dif-

ferences: for *P. pachydermus* [Czech Republic and UK (AJ439513)], 16% (76 nt); for *T. similis* [Czech Republic and UK (AJ439523)], 1% (13 nt); and for *T. sparsus* ('A' and 'B'), 20% (220 nt).

ITS2 of rDNA

The ITS2 sequences were obtained from 13 samples. Sequences of *Nanidorus minor*, *P. porosus* 'C' from California, and *T. pakistanensis* 'A' from India showed a high level of similarity (>99%) with sequences of corresponding species from China. Sequences of *T. similis* from the Czech Republic and UK were also similar. Surprisingly, sequences of *T. primitivus* from the Czech Republic and UK differed by 37%, whereas in the D2–D3 sequences these samples differed only by 0·3%.

PCR-RFLP study

The results of RFLPs of the partial 18S rDNA for *Nanidorus, Paratrichodorus* and *Trichodorus* using four restriction enzymes are given in Figure 3. Lengths of restriction fragments after digestion of PCR products with six enzymes are presented in Table 3. Restriction of PCR products by *Bse*NI clearly distinguished *Nanidorus*, *Paratrichodorus* (except for *P. porosus* 'C') and *Trichodorus* from each other, in numbers and lengths of fragments (Table 3). Restriction of *P. porosus* 'C' by this enzyme resulted in two fragments of similar lengths for some *Trichodorus* species. However, *Ava*I clearly differentiated this species from all *Trichodorus*. Three species of *Nanidorus* are distinguished by *Sat*I, although the differences between *N. minor* and *N. renifer* are based on fragments of less than 60 bp, which may not be clearly visible on agarose gels. PCR-RFLP and virtual digestion of 18S rDNA sequences revealed that six enzymes differentiated *P. anemones*, *P. hispanicus* 'B', *P. porosus* 'C', *P. teres* 'A' and *Paratrichodorus* sp. 'C' from each other and other *Paratrichodorus* species. *Paratrichodorus hispanus* 'A', *P. macrostylus* and *P. pachydermus* 'B' were indistinguishable from each other by any of the enzymes, as well as *Paratrichodorus allius* from *P. teres* 'C'. The six enzymes differentiated most *Trichodorus* species, except for *T. beirensis, T. sparsus* 'A' and 'B', *T. viruliferus* and *Trichodorus* sp. 'A', which generated similar RFLP profiles.

The D2–D3 PCR-RFLP profiles generated by four enzymes for 12 species of trichodorids are given in Figure 4. Lengths of restriction fragments from RFLP for the D2–D3 fragment of the 28S rDNA for *Nanidorus, Paratrichodorus* and *Trichodorus* are presented in Table 4. The four restriction enzymes *Bse*NI, *Pst*I, *Pvu*II and *Rsa*I separated all valid and putative species. The results of PCR-RFLP analysis based on all enzymes studied were identical to those expected from *in silico* analysis.

Discussion

Diagnostics of trichodorid nematodes is often difficult because of high intra- and interspecific variability of many morphological and morphometrical characters. Results of phylogenetic and sequence analyses may provide additional criteria to help identify and delimit species. In this study, Bayesian inference was used for phylogenetical reconstruction and species delimiting. Although the results show agreement between molecular and morphological identification for many trichodorid species, identification of some samples remains uncertain because of the presence of different sequences under the same specific name in GenBank. In this study a letter code was assigned for samples clustered separately in phylogenetic trees and morphologically identified as representatives of a single trichodorid species.

Phylogenetic and sequence analysis of the partial 18S and 28S rRNA gene sequences revealed a group of related sequences morphologically identified as representatives of the species *T. sparsus* (types 'A', 'B', 'C', 'D'). A high level of variation of morphological and morphometric characters has been reported between populations of *T. sparsus* from different countries (Loof, 1973; Peneva, 1988; Decraemer, 1995). Recently, Decraemer *et al.* (2008) distinguished three morphotypes of *T. sparsus* from Serbia based upon a combination of morphological

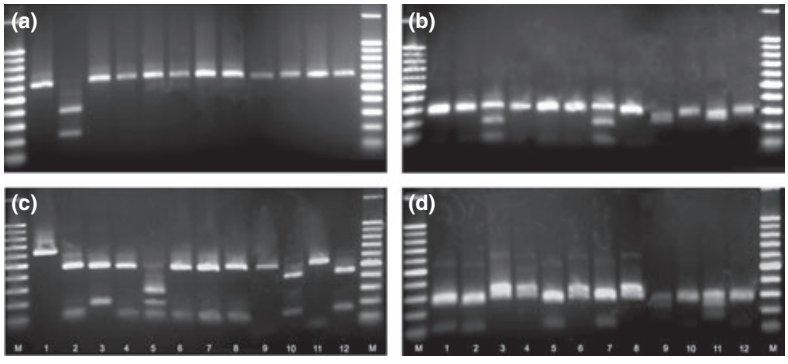

Figure 3 PCR-RFLP of the partial 18S rRNA gene for trichodorid nematodes. (a), *Taq*I; (b), *Sat*I; (c), *Bse*NI; (d), *Tsc*AI. Lanes: M, 100 bp DNA marker (Promega); 1, *Paratrichodorus pachydermus* 'B'; 2, *Trichodorus pakistanensis* 'A'; 3, *T. similis*; 4, *T. sparsus* 'A'; 5, *T. variopapillatus*; 6, *T. viruliferus*; 7, *T. primitivus*; 8, *T. sparsus* 'B'; 9, *Nanidorus renifer*; 10, *Trichodorus* sp. C; 11, *N. minor*; 12, *P. porosus* 'C'.

Table 3 Length (bp) of restriction fragments after digestion of PCR products obtained from the partial 18S rRNA gene for *Nanidorus, Paratrichodorus* and *Trichodorus*

Species	Unrestricted PCR[a]	Restriction enzyme					
		*Aat*II	*Ava*I	*Bse*NI (*Bsr*I)	*Sat*I (*Fnu*4HI)	*Taq*I	*Tsc*AI (*Tsp*RI)
Nanidorus minor	**610**	**610**	**610**	**546, 64**	**275, 240, 39, 36, 20**	**610**	**278, 217, 115**
Nanidorus renifer	**610**	610	610	**546, 64**	**275, 240, 56, 39**	**610**	**278, 217, 115**
Nanidorus nanus	610	610	610	546, 64	240, 214, 61, 56, 39	610	278, 217, 115
Paratrichodorus allius	**612**	**354, 258**	**463, 149**	**612**	**298, 275, 39**	**612**	280, 246, 86
Paratrichodorus anemones	**611**	**353, 258**	**611**	**611**	**297, 275, 39**	**526, 85**	**332, 279**
Paratrichodorus divergens	**612**	**354, 258**	**612**	**612**	**298, 275, 39**	**361, 251**	246, 220, 86, 60
Paratrichodorus hispanus 'A'	612	612	612	612	298, 275, 39	612	280, 246, 86
Paratrichodorus hispanus 'B'	**612**	**354, 258**	**612**	**612**	**298, 275, 39**	**612**	280, 246, 86
Paratrichodorus macrostylus	612	612	612	612	298, 275, 39	612	280, 246, 86
Paratrichodorus pachydermus 'B'	**612**	**612**	**612**	**612**	**298, 275, 39**	**612**	**280, 246, 86**
Paratrichodorus porosus 'C'	**612**	**612**	**463, 149**	**446, 166**	**298, 275, 39**	**612**	**280, 246, 86**
Paratrichodorus teres 'A'	612	612	612	612	298, 275, 39	558, 54	280, 246, 86
Paratrichodorus teres 'C'	612	354, 258	463, 149	612	298, 275, 39	612	280, 246, 86
Paratrichodorus sp. B	612	354, 258	612	612	298, 275, 39	361, 251	246, 220, 86, 60
Paratrichodorus sp. C	**612**	**354, 258**	**612**	**612**	**275, 241, 46, 39, 11**	**251, 246, 115**	280, 246, 86
Trichodorus beirensis	**612**	**354, 258**	**612**	**446, 100, 66**	**298, 275, 39**	**612**	332, 280
Trichodorus cylindricus 'A'	612	354, 258	612	446, 100, 66	275, 275, 39, 23	612	280, 246, 86
Trichodorus cylindricus 'B'	612	354, 258	612	446, 166	298, 192, 83, 39	612	280, 207, 86, 39
Trichodorus lusitanicus	**612**	**612**	**612**	**446, 100, 66**	**298, 275, 39**	**612**	332, 280
Trichodorus nanjingensis	612	354, 258	612	219, 215, 100, 66, 12,	298, 275, 39	612	332, 280
Trichodorus pakistanensis 'A'	**612**	354, 258	612	**446, 100, 66**	**298, 275, 39**	**388, 224**	**280, 246, 86**
Trichodorus pakistanensis 'B'	612	354, 258	612	215, 219, 100, 66, 12	298, 192, 83, 39	612	280, 246, 86
Trichodorus primitivus	**612**	**354, 258**	**612**	**446, 100, 66**	**298, 192, 83, 39**	**612**	**280, 246, 86**
Trichodorus similis	**612**	354, 258	612	**446, 166**	**298, 192, 83, 39**	**612**	**332, 280**
Trichodorus sparsus 'A'	**612**	354, 258	612	**446, 100, 66**	**298, 275, 39**	**612**	**332, 280**
Trichodorus sparsus 'B'	**612**	354, 258	612	**446, 100, 66**	**298, 275, 39**	**612**	**332, 280**
Trichodorus sparsus 'C'	612	354, 258	612	446, 100, 66	275, 241, 57, 39	612	332, 280
Trichodorus sparsus 'D'	612	612	612	446, 166	298, 275, 39	612	332, 280
Trichodorus variopapillatus	**612**	354, 258	612	**263, 183, 100, 66**	**298, 275, 39**	**612**	**280, 246, 86**
Trichodorus viruliferus	**612**	354, 258	612	**446, 100, 66**	**298, 275, 39**	**612**	**332, 280**
Trichodorus sp. A	**612**	**354, 258**	**612**	**446, 100, 66**	**298, 275, 39**	**612**	332, 280
Trichodorus sp. B	**612**	**354, 258**	**612**	**446, 100, 66**	**298, 192, 83, 39**	**612**	**332, 280**
Trichodorus sp. C	**611**	354, 257	611	**445, 116**	**298, 274, 39**	**611**	**280, 245, 86**

[a]Bold numbers – fragment verified by PCR-RFLP in Duarte *et al.* (2010) and/or this study.

characters and morphometrics, and noticed that separation between different morphotypes was not straightforward. Probably, the *T. sparsus* group consists of several sibling (or cryptic) species or subspecies. Final identification of *T. sparsus* isolates and other samples marked in this study as *T. pakistanensis* 'A', 'B', *P. porosus* 'A', 'B', 'C' and *P. pachydermus* 'A', 'B' will be possible after a more thorough morphological characterization of populations. Collection and molecular characterization of nematode materials from the type localities may be critical to resolving these identification problems. Species delimitation is controversial and should rely on the consensus of several data sets and criteria. Additional morphological, molecular and biogeographical data should be used to confirm the delimitation of species made in this and other studies.

PCR-RFLP analysis of ribosomal RNA gene sequences is one of the most effective methods of identification of different nematode groups comprising many species. The PCR-RFLP technique is a simple, rapid and cost-effective technique in comparison with other techniques. Because it is assumed that the sequence of the rRNA gene is conserved within a species, but diverse between species, selection of an appropriate gene marker for identification is crucial in developing a diagnostic. Duarte *et al.* (2011) designed a PCR-RFLP assay for identification of 12 trichodorids using a fragment of the 18S rRNA gene. The present analysis showed that not all species can be identified using this region. The D2–D3 of the 28S rRNA gene has higher interspecific variation and evolves more rapidly than the 18S rRNA gene, and therefore appears to be a more appropriate marker for identification of multiple species when compared with the 18S rRNA gene. Restriction of D2–D3 amplicons by four enzymes produces species-specific restriction patterns for all species analysed. Even though this study demonstrates advantages of PCR-RFLP analysis for the identification of stubby nematodes, ribosomal RNA gene markers may exhibit intraspecific

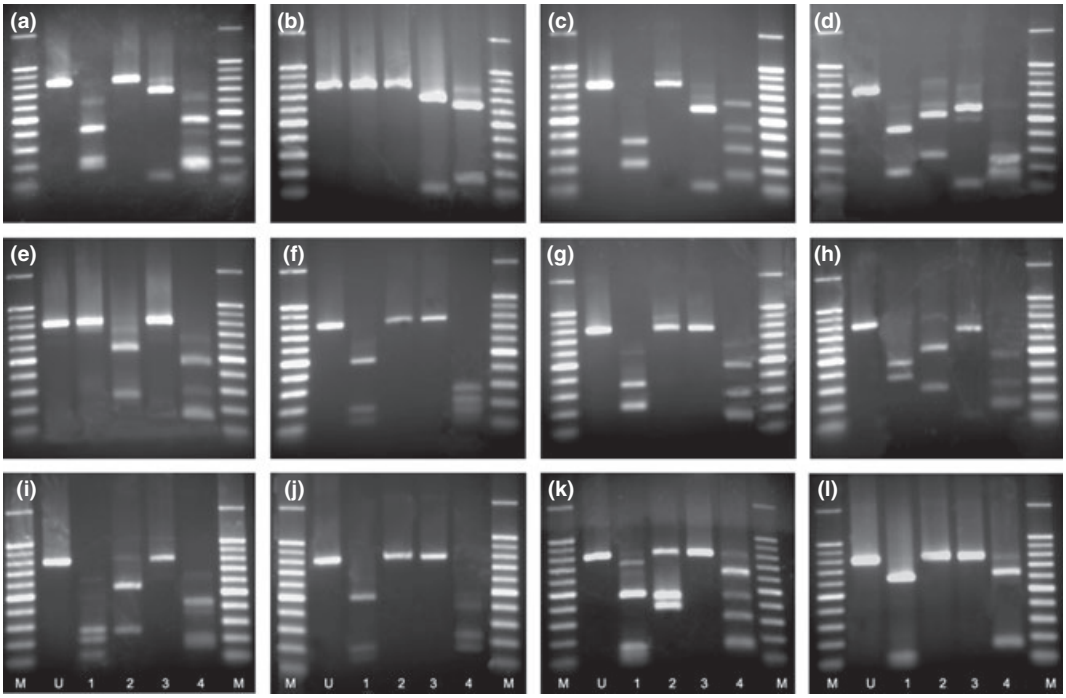

Figure 4 PCR-RFLP of the D2–D3 of 28S rRNA gene for trichodorid nematodes. (a), *Nanidorus minor*; (b), *N. renifer*; (c), *Paratrichodorus pachydermus* 'B'; (d), *P. porosus* 'C'; (e), *Trichodorus pakistanensis* 'A'; (f), *T. primitivus*; (g), *T. sparsus* 'A'; (h), *T. sparsus* 'B'; I, *T. variopapillatus*; (j), *T. viruliferus*; (k), *Trichodorus* sp. C; (l), *Trichodorus* sp. D. Lanes: M, 100 bp DNA marker (Promega); U, unrestricted PCR product; 1, *Bse*NI; 2, *Pst*I; 3, *Pvu*II; 4, *Rsa*I.

Table 4 Length (bp) of restriction fragments after digestion of PCR products obtained from the D2–D3 of the 28S rRNA gene for *Nanidorus, Paratrichodorus* and *Trichodorus*

		Restriction enzymes			
Species	Unrestricted PCR[a]	*Bse*NI	*Pst*I	*Pvu*II	*Rsa*I
Nanidorus minor	**800**	**405, 214, 181**	**800**	**691, 109**	**444, 189, 161, 6**
Nanidorus renifer	**797**	**797**	**797**	**688, 109**	**630, 161, 6**
Paratrichodorus anemones	794	680, 114	794	794	627, 161, 6
Paratrichodorus pachydermus 'A'	796	796	796	734, 62	501, 161, 128, 6
Paratrichodorus pachydermus 'B'	**796**	**348, 234, 214**	**796**	**583, 110, 103**	**629, 161, 6**
Paratrichodorus porosus 'C'	**795**	**441, 182, 172**	**529, 266**	**581, 110, 104**	**246, 223, 161, 128, 21, 6**
Trichodorus cedarus	797	797	797	400, 397	630, 161, 6
Trichodorus cylindricus	797	483, 314	547, 250	797	472, 161, 158, 6
Trichodorus nanjingensis	796	*796*, 516, 280	796	686, 110	354, 161, 275, 6
Trichodorus pakistanensis 'A'	**797**	**797**	**547, 250**	**797**	**472, 161, 158, 6**
Trichodorus primitivus	**796**	**455, 181, 121, 12**	**796**	**796**	**286, 227, 161, 116, 6**
Trichodorus similis	796	**482, 314**	**796**	**796**	402, 227, 161, 6
Trichodorus sparsus 'A'	**796**	**350, 232, 214**	**796**	**796**	**471, 161, 158, 6**
Trichodorus sparsus 'B'	**796**	**446, 350**	**530, 266**	**686, 110**	**471, 161, 158, 6**
Trichodorus variopapillatus	**796**	**268, 214, 133, 115, 66**	**546, 250**	**734, 62**	**419, 210, 161, 6**
Trichodorus viruliferus	**794**	**480, 181, 133**	**794**	**794**	**243, 226, 161, 158, 6**
Trichodorus sp. C	**797**	**455, 161, 115, 66**	*797*, 431, 366	**797**	**630, 161, 6**[b]
Trichodorus sp. D	**796**	**616, 115, 65**	**796**	**796**	**629, 161, 6**[b]
Trichodorus sp. E	798	315, 233, 214, 36	548, 250	798	260, 222, 161, 149, 6

[a]Bold number – fragment verified by PCR-RFLP in this study; italics number – addition fragments.
[b]Presence of several additional bands on a gel not considered after virtual restriction.

polymorphism, which will cause variation in restriction maps in different geographically distinct populations. Thus, it will be necessary to investigate the variation of sequences from different geographically separated populations for some species before application of this technique.

Phylogenetic relationships within trichodorids were recently studied by Van Megen *et al.* (2009) and Duarte *et al.* (2010) using full length 18S rRNA sequences, with only 17 and 15 stubby nematode species included in these analyses, respectively. The present study, which included more species and compared a smaller fragment of the 18S rRNA gene, showed similar relationships between taxa as previous studies. However, this analysis generated low supports for relationships between major clades and also revealed artifactual paraphylies for *Paratrichodorus* and *Nanidorus*. Major differences in topologies between trees were observed mainly in positions of poorly supported clades. Observed paraphyly for *Trichodorus* in D2–D3 of the 28S rRNA gene tree here and the 18S rRNA gene tree by Van Megen *et al.* (2009), and for *Paratrichodorus* and *Nanidorus* in the 18S rRNA gene tree here, might be explained by increasing for absolute total number of species on the branches and nodes with more descendent species, which lead to an unbalanced tree. The increase in imbalance is consistent with a cumulative effect of differences in diversification rates between branches (Holman, 2005). Validity of the genus *Nanidorus* has been the subject of some debate (Siddiqi, 1980, 2002) and only recently Duarte *et al.* (2010) proposed accepting *Nanidorus* as a valid genus based on morphological features and the results of molecular analysis of the 18S rRNA gene. Although the 18S rRNA gene analysis in the present study does not generate enough resolution to understand relationships between *Nanidorus*, the D2–D3 of the 28S rRNA gene, having a higher phylogenetic signal, gives clear evidence for its monophyly and also confirms closer relationships of this genus with the genus *Trichodorus* rather than with *Paratrichodorus*.

Observed clustering of trichodorid species in the tree was generally in agreement with reported phenotypic similarity of male characters (Decraemer, 1995; Decraemer & Baujard, 1998). Sorting of three prime characters (number of ventromedian precloacal supplements, number of ventromedian cervical pappillae and habitus), Decraemer & Baujard (1998) distinguished 13 male groups within Trichodoridae. In the trees here, *T. lusitanicus, T. viruliferus, T. beirensis, T. similis, T. primitivus* and *T. variopapillatus,* all belonging to the male Group 12, and *T. cylindricus* from Group 11, clustered together. *Trichodorus sparsus* (Group 10) was in a separate clade on D2–D3 of the 28S rRNA and 18S RNA gene trees. Two major subclades of *Paratrichodorus* on the 18S rRNA gene tree also corresponded to the male groupings: (i) *P. allius* (Group 2), *P. porosus* (Group 3), *P. teres* (Group 4); and (ii) *P. anemones, P. pachydermus, P. hispanus, P. macrostylus* (Group 6).

Thus, PCR-RFLP and sequencing of ribosomal RNA markers appear to be a useful and appropriate method for characterization and accurate identification of stubby nematodes. However, larger numbers of species and populations from diverse origins and other alternative gene markers should be included and analysed in future studies to confirm the findings made in this work.

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References

Blaxter ML, De Ley P, James R *et al.*, 1998. A molecular evolutionary framework for the phylum Nematoda. *Nature* **392**, 71–5.

Boutsika K, Brown DJF, Philips MS, Blok VC, 2004a. Molecular characterisation of the ribosomal DNA of *Paratrichodorus macrostylus*, *P. pachydermus*, *Trichodorus primitivus* and *T. similis* (Nematoda: Trichodoridae). *Nematology* **6**, 641–54.

Boutsika K, Phillips MS, MacFarlane SA, Brown DJF, Holeva RC, Blok VC, 2004b. Molecular diagnostics of some trichodorid nematodes and associated *Tobacco rattle virus*. *Plant Pathology* **53**, 110–6.

Brown DJF, Boag B, 1988. An examination of methods used to extract virus-vector nematodes (Nematoda: Longidoridae and Trichodoridae) from soil samples. *Nematologia Mediterranea* **16**, 93–9.

Coolen WA, 1979. Methods for the extraction of *Meloidogyne* spp. and other nematodes from roots and soil. In: Lambetrti F, Taylor CE, eds. *Root-knot Nematodes (Meloidogyne species): Systematics, Biology and Control*. London, UK: Academic Press, 317–29.

Decraemer W, 1995. *The Family Trichodoridae: Stubby Root and Virus Vector Nematodes*. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Decraemer W, Baujard P, 1998. A polytomous key for the identification of species of the family Trichodoridae Thorne, 1935 (Nematoda: Triplonchida). *Fundamental and Applied Nematology* **21**, 37–62.

Decraemer W, Robbins RT, 2007. The who, what and where of Longidoridae and Trichodoridae. *Journal of Nematology* **39**, 295–7.

Decraemer W, Radivojevic M, De La Pena E, 2008. Trichodoridae (Nematoda: Triplonchida) from the Tara National Park, Serbia, and proposal of *Trichodorus pseudobursatus* n. sp. *Nematology* **10**, 405–31.

Duarte IM, De Almeida MTM, Brown DJF, Marques I, Neilson R, Decraemer W, 2010. Phylogenetic relationships, based on SSU rDNA sequences, among the didelphic genera of the family Tridhodoridae from Portugal. *Nematology* **12**, 171–80.

Duarte IM, Almeida MTM, Duarte MM, Brown DJF, Neilson R, 2011. Molecular diagnosis of trichodorid species from Portugal. *Plant Pathology* **60**, 586–94.

Holeva R, Phillips MS, Neilson R *et al.*, 2006. Real-time PCR detection and quantification of vector trichodorid nematodes and Tobacco rattle virus. *Molecular and Cellular Probes* **20**, 203–11.

Holman EW, 2005. Nodes in phylogenetic trees: The relation between imbalance and number of descendent species. *Systematic Biology* **54**, 895–9.

Hooper DJ, 1986a. Extraction of free-living stages from soil. In: Southey JF, ed. *Laboratory Methods for Work with Plant and Soil Nematodes*. London, UK: Her Majesty's Stationery Office, 5–30.

Hooper DJ, 1986b. Handling, fixing, staining and mounting nematodes. In: Southey JF, ed. *Laboratory Methods for Work with Plant and Soil Nematodes*. London, UK: Her Majesty's Stationery Office, 59–80.

Huelsenbeck JP, Ronquist F, 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–5.

Kumari S, 2010. Description of *Paratrichodorus pachydermus* (Nematoda: Trichodoridae) from the Czech Republic. *Helminthologia* **47**, 196–8.

Kumari S, Decraemer W, 2009. First report of *Trichodorus variopapillatus* (Nematoda: Trichodoridae) from the Czech Republic. *Plant Disease* **93**, 966.

Kumari S, Decraemer W, 2011. First report of *Trichodorus primitivus*, *T. sparsus* and *T. viruliferus* (Nematoda: Trichodoridae) from the Czech Republic. *Helminthologia* **48**, 195–9.

Kumari S, Vohanka J, Decreamer W, 2007. First report of *Trichodorus similis* from the Czech Republic (Nematoda: Trichodoridae). *Plant Disease* **91**, 228.

Loof PAA, 1973. Taxonomy of the *Trichodorus aequalis* complex (Diphtherophorina). *Nematologica* **19**, 49–61.

Nunn GB, 1992. *Nematode Molecular Evolution*. Nottingham, UK: University of Nottingham, PhD thesis.

Peneva V, 1988. Nematodes of the fam. Trichodoridae (Diphtherophorina) from forest nurseries in Bulgaria. *Khelmintologiya* **26**, 47–66.

Riga E, Karanastasi E, Oliveira CMG, Neilson R, 2007. Molecular identification of two stubby root nematode species. *American Journal of Potato Research* **84**, 161–7.

Siddiqi MR, 1980. On the generic status of *Atlantadorus* Siddiqi, 1974 and *Nanidorus* Siddiqi, 1974 (Nematoda: Trichodoridae). *Systematic Parasitology* **1**, 151–2.

Siddiqi MR, 2002. *Ecuadorus equatorius* gen. n., sp. n. and *Nanidorus mexicanus* sp. n. (Nematoda: Trichodoridae). *International Journal of Nematology* **12**, 197–202.

Sites JW, Marshall JC, 2004. Operational criteria for delimiting species. *Annual Review of Ecology, Evolution and Systematics* **35**, 199–227.

Stanton JM, McNicol CD, Steele V, 1998. Non-manual lysis of second-stage *Meloidogyne* juveniles for identification of pure and mixed samples based on the polymerase chain reaction. *Australasian Plant Pathology* **27**, 112–5.

Swofford DL, 2003. *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4·0b 10*. Sunderland, MA, USA: Sinauer Associates.

Tanha Maafi Z, Subbotin SA, Moens M, 2003. Molecular identification of cyst-forming nematodes (Heteroderidae) from Iran and a phylogeny based on the ITS sequences of rDNA. *Nematology* **5**, 99–111.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The clustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**, 4876–82.

Van Megen H, Elsen SVD, Holterman M *et al.*, 2009. A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences. *Nematology* **11**, 927–50.

Vrain TC, Wakarchuk DA, Levesque AC, Hamilton RI, 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* **15**, 563–73.