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Length variation and repetitive sequences of Internal Transcribed Spacer of ribosomal RNA gene, diagnostics and relationships of populations of potato rot nematode, *Ditylenchus destructor* Thorne, 1945 (Tylenchida: Anguinidae)

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Summary – Seventy-eight ITS rRNA gene sequences obtained from the potato rot nematode, *Ditylenchus destructor*, collected across the world from different hosts were compared and analysed. The ITS rRNA gene sequences showed significant length variation between populations. The differences in this rRNA fragment length were due to the presence of repetitive elements in the ITS1, which were characterised by relatively higher rates of substitution changes. Reconstruction of secondary structure for the ITS1 revealed that these minisatellites formed a stem structure. Phylogenetic analyses of ITS rRNA and D2-D3 expansion segments of 28S rRNA gene sequences showed that all studied populations clustered in two major clades: a group of populations having the ITS sequences with the repetitive elements and a group of populations without the repetitive elements in the ITS. We propose to distinguish seven ITS rRNA haplotypes within potato rot nematode populations. PCR-ITS-RFLP diagnostic profiles are presented for these ITS haplotypes and usefulness of recently developed PCR methods with species-specific primers for *D. destructor* are analysed and discussed.

Keywords – 5.8S rRNA, ITS1 rRNA, minisatellites, molecular, PCR-RFLP, phylogeny, secondary structure.

The potato rot nematode, *Ditylenchus destructor* Thorne, 1945, is a plant-parasitic nematode of great economic importance causing significant loss in production of potato, bulbous iris, sweet potato, sugar beet and several other crops. The potato rot nematode is a quarantine organism for many countries and regulatory organisations. *Ditylenchus destructor* is a polyphagous nematode infecting more than 100 species of plants from a wide variety of families. In the absence of host plants it is capable of feeding and reproducing on mycelia of many fungi. Although there are a number of reports on differences in host range, pathogenicity and virulence within the species, so far no biological races of *D. destructor* have been designated and characterised (Wu, 1960; Goodey, 1962; Hooper, 1973; Sturhan & Brzeski, 1991; Anon., 2008). Several molecular methods have been developed for diagnostics and characterisation of *D. destructor.* Wendt *et al.* (1993, 1995) first showed that amplification of ITS-rRNA gene by PCR followed by RFLP with several restriction enzymes could clearly distinguish *D. destructor* infecting potato plants from *D. dipsaci* (Kühn, 1857) Filipjev, 1936, *D. myceliophagus* Goodey, 1958 and *D. africanus* Wendt, Swart, Vrain & Webster, 1995. PCR-ITS-RFLP was successfully applied for identification of different isolates of *D. destructor* (Ji *et al.*, 2006; Liu *et al.*, 2007; Marek *et al.*, 2010). Several methods using PCR with specific primers

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designed based on specific combination of nucleotides in the ITS rRNA gene were also recently developed for the detection of this nematode (Liu *et al.*, 2007; Wan *et al.*, 2008; Marek *et al.*, 2010). The results of the analysis of PCR-ITS-RFLP and ITS-rRNA gene sequences (Ji *et al.*, 2006; Liu *et al.*, 2007; Wang *et al.*, 2007; Huang *et al.*, 2009; Xu *et al.*, 2009), however, revealed significant variations between different isolates in length and base composition. Moreover, several short and long repeated DNA motifs were found in the ITS1 rRNA gene fragment of this species (Marek *et al.*, 2010).

Considering these facts, the present study was devoted to understanding the nature of the ITS-rRNA variation and its effect on results of molecular diagnostic tests for *D. destructor*. We also performed sequence and phylogenetic analysis to estimate sequence variation and reconstruct relationships between populations from different hosts and geographical regions. A table of restriction profiles after virtual digestion of the ITS sequences for different population groups is given.

Materials and methods

Nematode materials

Nematodes were extracted from potatoes (*Solanum tuberosum* L.) collected in Russia from three regions: Moscow (CA210), Nizhnii Novgorod (CA205, CA215) and Jaroslavl (CA213); and from Iran in four locations: Damavand, Tehran province (CA206); Ardabil, Ardabil province (CA211); Daryon, Fars province (CA212) and Qorveh, Kurdistan province (CA214). Nematodes were also extracted from sweet potato, *Ipomoea batatas* (L.) Lam, collected in China: Weifang, Shandong province and Luoyang, Henan province. One population of *D. destructor*, kindly provided by D. Harshman, originated from Wisconsin and was maintained in a fungal culture at Clemson University, Clemson, SC, USA.

DNA extraction, PCR, cloning and sequencing

Several nematode specimens from each population were put into a drop of water and cut by a scalpel under a binocular microscope. Nematode remains were transferred into an Eppendorf tube containing 16 μl ddH_2O, 2 μl of 10× PCR buffer (Qiagen, Valencia, CA, USA), 2 μl proteinase K (600 μg ml^{-1}) (Promega, Madison, WI, USA). The tube was then incubated at 65°C (1 h) and 95°C (10 min). Extracted DNA (2 μl) was transferred into a 0.2 ml Eppendorf tube containing: 2.5 μl 10× *Taq* incubation buffer, 5 μl Q solution, 0.5 μl dNTPs mixture (*Taq* PCR Core Kit, Qiagen), 0.15 μl of each primer (1.0 μg μl^{-1}), 0.1 μl *Taq* polymerase and double distilled water to a final volume of 25 μl. The forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and the reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGG GT-3′) amplifying the ITS1-5.8S-ITS2 of rRNA and forward D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and reverse D3B (5′-TCGGAAGGAACCAGCTACTA-3′) amplifying the D2-D3 expansion segments of 28S rRNA were used in PCR. The PCR amplification profile consisted of 4 min at 94°C; 35 cycles of 1 min at 94°C, 1.5 min at 55°C and 2 min at 72°C, followed by a final step of 10 min at 72°C. Two μl of the PCR product was run on a 1% TAE buffered agarose gel. The PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen), cloned into the pGEM-T vector and transformed into JM109 High Efficiency Competent Cells (Promega). Several clones of each sample were isolated using blue/white selection and submitted to PCR with same primers. PCR products from each clone were sequenced in both directions. New sequences obtained in the present study were submitted to the GenBank database under the accession numbers HQ235675-HQ235698.

RFLP-rRNA

Of the ITS or D2-D3 purified product 3-5 μl was digested by one of following restriction enzymes: *Bsh*143I, *Bsh*1236I, *Dde*I, *Hin*fI, *Nde*I, *Pst*I, *Rsa*I, *Sdu*I or *Tru*9I (*Mse*I) in the buffer stipulated by the manufacturer. The digested DNA was run on a 1% TAE buffered agarose gel, stained with ethidium bromide, visualised on a UV transilluminator and photographed. The exact length of each restriction fragment from the PCR products was obtained by a virtual digestion of the sequence using WebCutter 2.0 (www.firstmarket.com/cutter/cut2.html).

PCR with species-specific primer

Several *D. destructor* samples and a *D. dipsaci* sample were used to test a species-specific primer developed by Liu *et al*. (2007). PCR mixture was prepared as described above. The D2 (5′-TGGATCACTCGGCGGCTCGTA GA-3′) and D1 (5′-ACTGCTCTGCGTTTGGCTTCA-3′) primers were used in PCR. The PCR amplification profile consisted of 4 min at 94°C; 30 cycles of 1 min at 94°C, 45 s at 57°C and 45 s at 72°C, followed by a fi-

nal step of 10 min at 72°C. Of the PCR product 2 μl was run on a 1.2% TAE buffered agarose gel, stained and photographed.

Sequence alignment and secondary structure reconstruction for ITS rRNA

Newly obtained DNA sequences of *D. destructor* were aligned with other available sequences in GenBank using ClustalX 1.83 with default options (Thompson *et al.*, 1997). ITS-rRNA gene sequences for *D. destructor* submitted by Powers *et al.* (2001), Subbotin *et al.* (2005), Wang *et al.* (2007), Marek *et al.* (2010), Bao *et al.* (unpubl.), Guo *et al*. (unpubl.), Peng *et al*. (unpubl.), Xu and Xie (unpubl.), Yu and Peng (unpubl.), Zhang *et al*. (unpubl.) and *D. myceliophagus* selected as an outgroup taxon were used in the present study. D2-D3 of 28S rRNA sequence of Iranian sample were aligned with sequences submitted by Peng *et al.* (unpubl.) and two tylenchids used as outgroup taxa. Mfold software Version 3 (Zuker, 2003) (http://frontend.bioinfo.rpi.edu/applications/mfold/) was applied to predict the secondary structure for ITS1, 5.8S and ITS2 using the energy minimisation approach. Secondary structure of 5.8 rRNA was optimised using a universal model of this molecule proposed by Vaughn *et al.* (1984). Structures were visualised using Varna (Darty *et al.*, 2009) and drawn with Photoshop 7.0. Final alignment was reconstructed manually with the GenDoc program (Nicholas *et al.*, 1997), taking into consideration the secondary structure of helices.

Sequence analysis and phylogenetic analysis

Pairwise divergence between taxa was calculated using PAUP* 4b10 (Swofford, 2003) from the ITS1-5.8S-ITS2 sequence alignment as the absolute distance value and the percent mean distance. The boundaries of regions containing repetitive sequence were designated by a dotplot analysis as implemented by Dot Plots program in Molecular Toolkit (http://arbl.cvmbs.colostate.edu/molkit) with window size equal to 11 and mismatch limit equal to 3. By plotting the sequence against itself, the repetitive region becomes visible as sets of short parallel lines. To reveal consensus sequences for repetition and number of their copies within the ITS1 for each sequence sample we used the Tandem Repeats Finder Version 4.0 (Benson, 1999) with the default settings.

The sequence datasets were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The best fit model of DNA evolution for BI was obtained using the program MrModeltest 2.2 (Nylander, 2002) with the Akaike Information Criterion in conjunction with PAUP*. BI analysis under the GTR + I + G model was initiated with a random starting tree and run with the four Metropolis-coupled Markov chain Monte Carlo (MCMC) for 10^6 generations. The MCMC were sampled at intervals of 100 generations. The log-likelihood values of the sample points stabilised after approximately 10^3 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) more than 70% are given for appropriate clades.

Results

ITS length variation and characterisation of repetitive sequences

PCR products of the ITS region for studied *Ditylenchus* samples ranged approximately from 720 to 915 bp. Single PCR products were obtained from all studied samples except for several isolates from Russia (CA205, CA210, CA213), which generated two bands on a gel. Seventy-eight sequences of *D. destructor*, 23 of which were newly obtained in this study, and two sequences of *D. myceliophagus* were analysed. In *D. destructor*, ITS1 varied in length from 315 to 473 bp, 5.8S measured *ca* 154 bp and ITS2 was 207 bp. The length variation observed in the ITS1 in some *D. destructor* was caused by an insertion varying in length from 57 to 188 bp. Sequence divergence within all *D. destructor* samples reached 5.6% (33 nt) and after exclusion of an insertion from the analysis −2.6% (18 nt). It was maximal between sequences having an insertion, whereas maximal divergence within sequences without insertion was 0.8% (6 nt). Diversity of insertion sequences only reached 16.6% (27 nt).

Dotplot analyses show that ITS1-5.8S-ITS2 sequences contained a repeated motif with a few nucleotides across the whole length and long length repetitive elements in the ITS1 region (Fig. 1). Tandem Repeat Finder software revealed two repeats: *i*) the first with consensus sequence containing 13 nt: $(KCTRTGTRCYTGC)_n$; and *ii*) the second repeat with consensus sequence containing 12 nt: $(GCTYKYATTWGH)_n$. In the insertion these core sequences were found two, four or five times. A BLAST search on the nucleotide databases of Genbank showed that these minisatellites were unique for *D. destructor*.

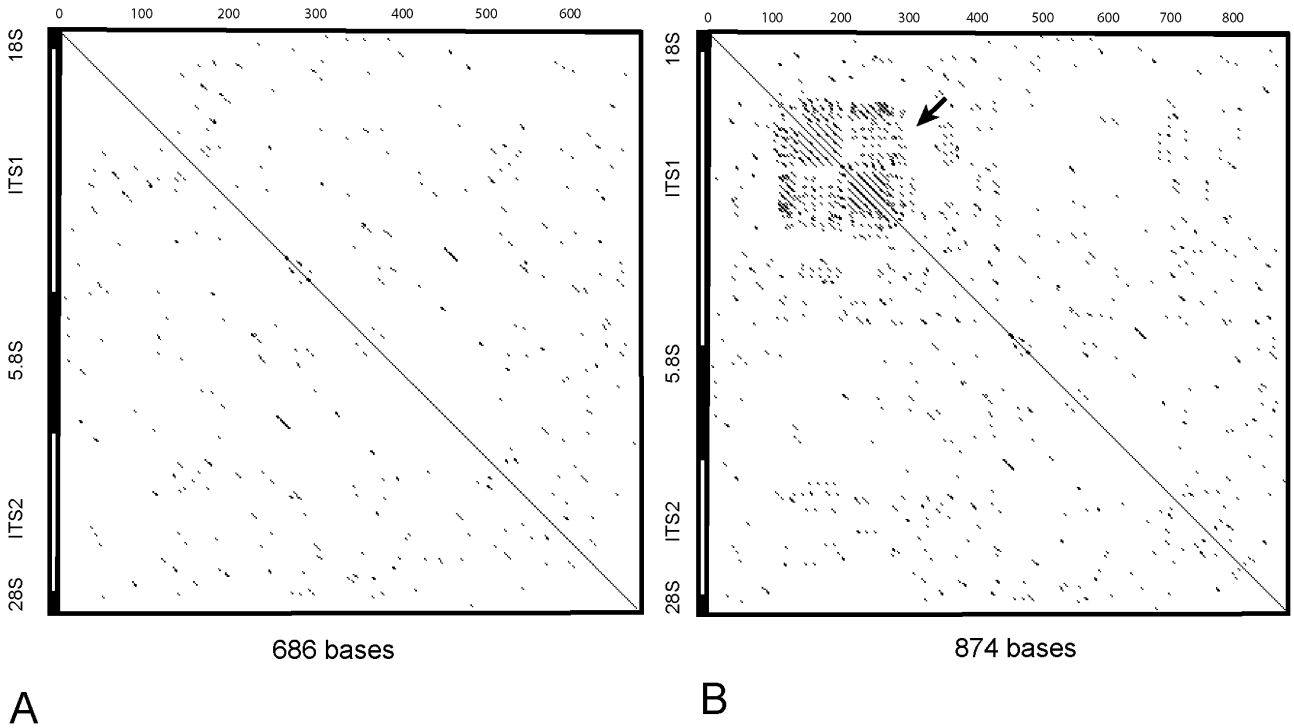


Fig. 1. *Dotplot analyses of the 18S-ITS1-5.8S-ITS2-28S rRNA gene sequences for* Ditylenchus destructor *without (A) and with (B) insertion. Arrow shows repeated regions in the ITS1. The sequences under the GenBank accession numbers EU499915 and FJ911551 were used for this analysis.*

Secondary structure prediction of the insertions showed the minisatellite region was folded in a stem-like structure named as helix H9 in the ITS1 (Figs 2, 3, 4A, B). The second repeat was a reversed complement of the first one and they were able to form a stable secondary structure of the helix H9 (Fig. 4A). Based on effectiveness of mutations in minisatellite region, the helix H9 can be divided into two groups: *i*) types I, II and III; and *ii*) types IV, V and VI (Fig. 4B).

Putative secondary structures for ITS1 and ITS2 rRNA

Mfold 3.0 software reconstructed optimal secondary structures for the 3′-end of 18S rRNA-ITS1 and the 3′-end of 5.8S-ITS2-5′end of 28S for *D. destructor* as shown in Figures 2, 3A and 3D, respectively. Considering the fact that the first 15 nucleotides in the 5′-end of ITS1 belonged to the 18S rRNA gene, the ITS1 itself represents three domain structures for *D. destructor*: *i*) helix H3; *ii*) helices H5-H9; and *iii*) helices H10-H14; and four domain structures for *D. myceliophagus*: *i*) helix H3; *ii*) helix H4; *iii*) helices H5-H8; and *iv*) helices H10-H14. The structure of the ITS1 was conservative for *D. destructor* and differed between samples in the presence/absence and length of helix H9 and lengths of helices H8 and H11. In samples having short ITS1 sequences, helix H9 was absent (Fig. 3A), while in samples with long ITS1 sequences, interior loop I3 was present (Fig. 3B, C). The long H8 helix was in two sequences: DQ471335 and HQ235677. Deletion in helix H11 and terminal loop T8 was observed in sequence EF088930. Helix H4 (Figs 2A, 3A) was observed in *D. myceliophagus* only. Eight locations with point mutations observed at least in two studied sequences were found in the ITS1 without the helix H9 for *D. destructor.* Six types of helix H9 differing in base composition and sequence length were found among all studied *D. destructor* samples (Fig. 4).

The ITS2 represents three structural domain structures: *i*) helices H1 and H2; *ii*) helices H3-H5; and *iii*) helices H6-H11 (Fig. 3D). Eight locations with point mutations observed at least in two analysed sequences were found

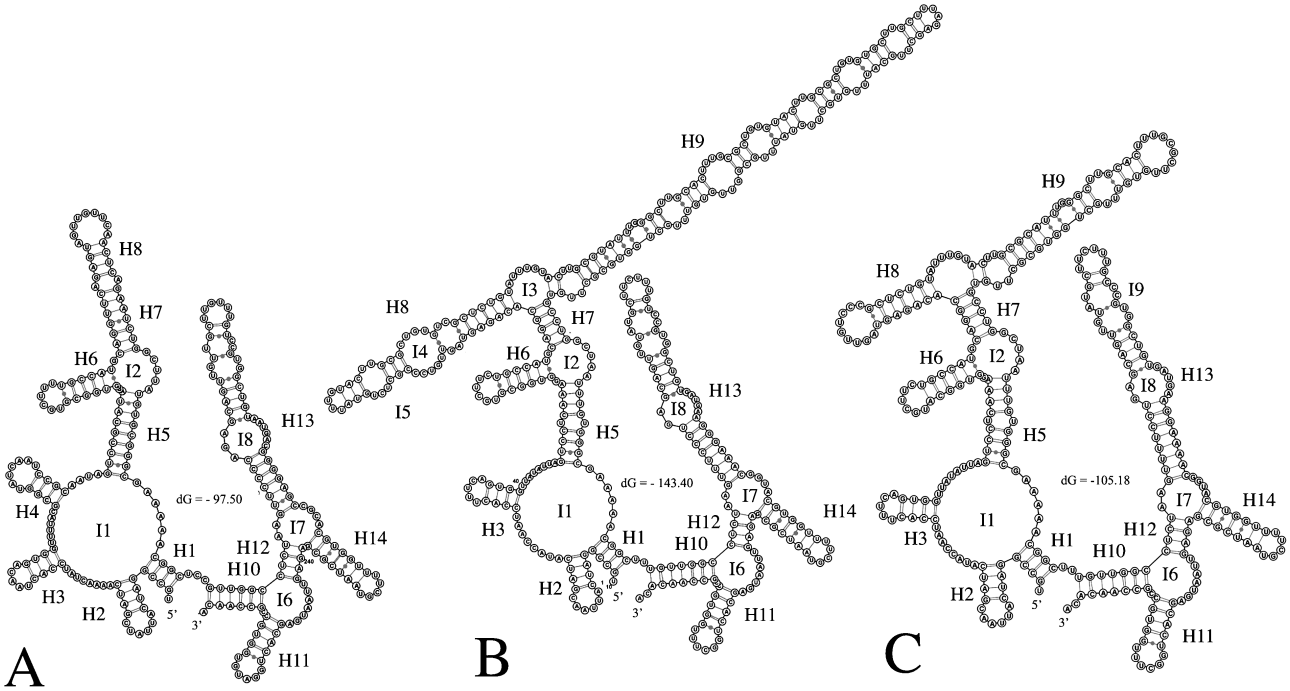


Fig. 2. *Putative secondary structures of the ITS1 and ITS2 for* Ditylenchus destructor *and* D. myceliophagus. *A:* D. myceliophagus*, DQ151458; B:* D. destructor*, DQ471335; C:* D. destructor*, HQ235689. Free energy levels (dG in kcal mol*$^{-1}$*) are given for three ITS1 structures.*

in the ITS2 for *D. destructor*. The models of ITS1 and ITS2 secondary structures suggested here are supported by compensatory and semi-compensatory base changes that maintain stability of structure.

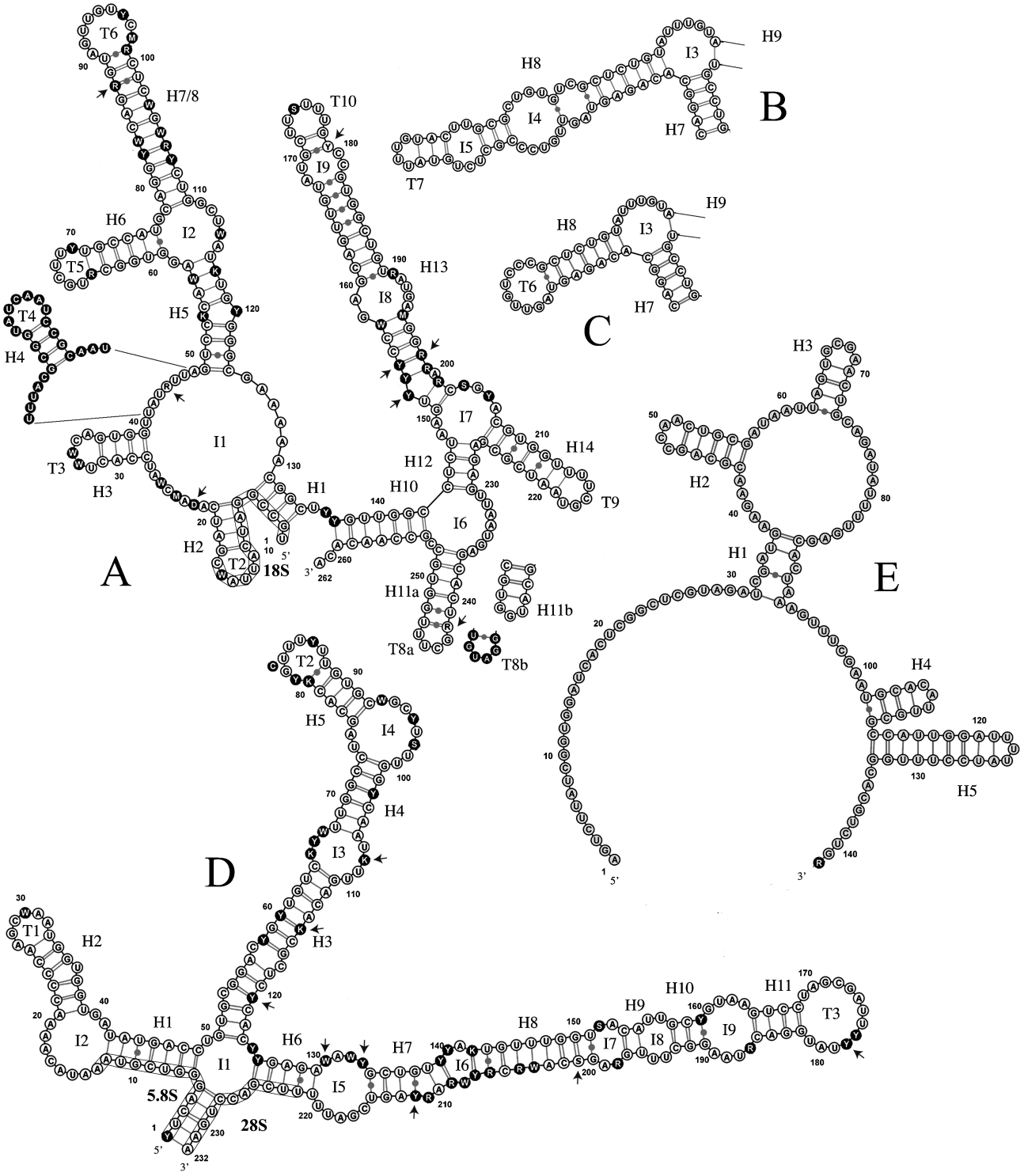
The secondary structure of the 5.8S rRNA shows four paired regions (Fig. 3E): first, a region with a basal pairing (H1) and an internal loop with two helices (H2 and H3), second and third, each with a single helix (H4 or H5). The fourth region includes the 5′-end of the 5.8S rRNA pairing with the 3′-end of the 28S rRNA (Fig. 3D). Only two point mutations observed at least in two analysed sequences were found between *D. destructor* and *D. myceliophagus* (Fig. 3D, E).

Classification and diagnostics of ITS haplotypes of *D. destructor*

Sequence analysis revealed at least seven distinct haplotypes of the ITS1-5.8S-ITS2 sequences among all studied samples. Following the proposal by Wang *et al*. (2007) and Wan *et al.* (2008), who first differentiated two ITS types (A and B) within *D. destructor*, we kept those types as haplotypes A and B and distinguished five additional haplotypes: C, D, E, F and G. Based on present knowledge, the distribution patterns of haplotypes are as follows: all haplotypes, except for type G, are found in China, types B, G and E are in Europe, and types C and E are in the USA. Types G and E were observed together in several populations collected in Russia. Haplotypes A, D and F were found in China only. Haplotypes A, B, C and E were reported from both potato and sweet potato, whereas haplotype F was found in sweet potato and haplotype G was found in potato only.

The sizes for restriction fragments generated by four diagnostic restriction enzymes for seven ITS haplotypes of *D. destructor* and *D. myceliophagus* are given in Table 1 and the diagnostic PCR-ITS-RFLP profiles for four haplotypes are presented in Figure 5. Combination of four restriction enzymes, *Dde*I, *Hin*fI, *Tru*9I and *Sdu*I, distinguished ITS haplotypes from each other.

Comparative sequence analysis of the ITS sequences and the species-specific primers for detection of *D. destructor* developed by Liu *et al*. (2007), Wan *et al.* (2008) and Marek *et al.* (2010) revealed that only the primer combination: D2 and D1 (expected PCR product size = 346 bp) proposed by Liu *et al*. (2007) can detect *D. destruc-*


A
B
C
D
E
18S
28S
5.8S
H1
H2
H3
H4
H5
H6
H7/8
H7
H8
H9
H10
H11
H11a
H11b
H12
H13
H14
I1
I2
I3
I4
I5
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T8a
T8b
T9
T10

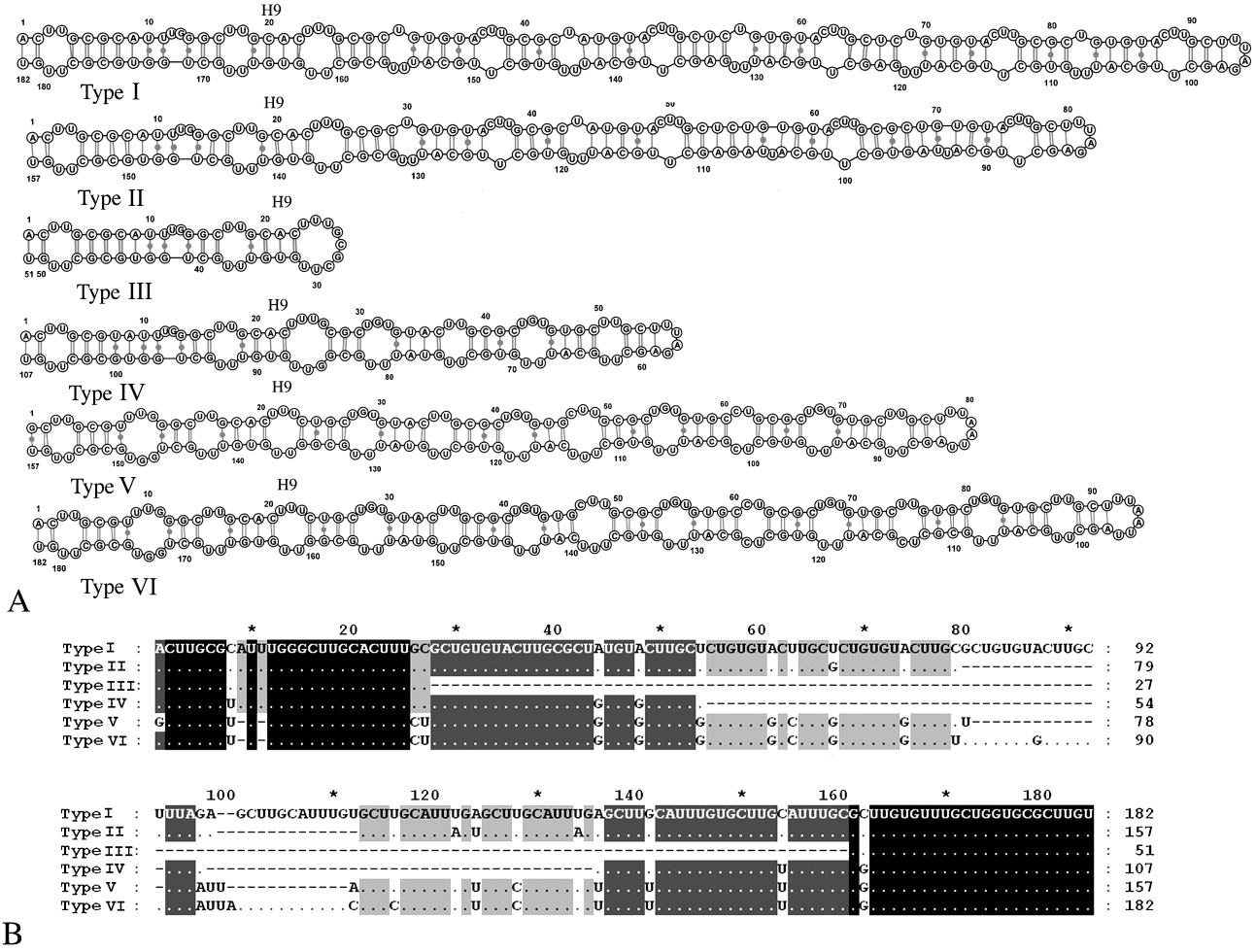

Fig. 4. *A: Types of helix H9 of ITS1; B: Alignment of helix H9 sequences.*

tor with all haplotypes. Results of PCR with this species-specific primer are given in Figure 6. PCR with specific primers developed by Wan *et al.* (2008) with combinations: DdS1 (5′-TCGTAGATCGATGAAGAACGC-3′) and DdS2 (5′-ATTATCTCGAGTGGGAGCGC-3′) (expected PCR product size = 252 bp) can identify haplotype A only; DdL1 (5′-TTGTGTTTGCTGGTGCGCTT GT-3′) and DdL2 (5′-GAGTGAGAGCGATGTCAACAT TG-3′) (expected PCR product size = 485 bp) detect all haplotypes, except for haplotype A. The primer combination Des2-F (5′-GTGCTTGTATTTGCGGTTGTG-3′) and Des1-R (5′-TGCTAGGCCAAAGAGACAGC-3′)

Fig. 3. *Putative secondary structures of the ITS1, 5.8S and ITS2 for* Ditylenchus destructor *and* D. myceliophagus*. A: Consensus ITS1 structure for* D. destructor *without helix H9 and* D. myceliophagus*. Helix H4 and the terminal loop T8b are given for* D. myceliophagus*; B: Fragments of ITS1 of* D. destructor *for DQ471335 showing helix H8 with an insertion; C: Consensus fragments of ITS1 of* D. destructor *having helix H9 and showing helices H7 and H8 with interior loop I3; D: Consensus ITS2 structure for* D. destructor *and* D. myceliophagus*. The first 15 nucleotides in the 5′-end of ITS1 are the 18S rRNA. The first 13 nucleotides in the 5′-end of ITS2 are 5.8S rRNA and the last 11 nucleotides in the 3′-end of ITS2 are the 28S rRNA; E: Fragment of secondary structure for 5.8S rRNA. Abbreviations: H = helix; I = interior loop; T = terminal loop. Arrows indicate point mutations observed in at least in two studied sequences for* D. destructor*. Nucleotides with a black background are point mutations for* D. myceliophagus*.*

Table 1. *Approximate sizes (in bp) of restriction fragments generated by virtual digestion by some diagnostic restriction enzymes for PCR products of the ITS-rRNA regions amplified by TW81 and AB28 primers for* Ditylenchus destructor *and* D. myceliophagus.

Enzyme	*D. destructor*							*D. myceliophagus*
	ITS haplotype (Helix H9 type)							
	A (absent)	B (H9-VI)	C (H9-I)	D (H9-II)	E (H9-III)	F (H9-IV)	G (H9-V)	
Unrestricted PCR	727	915	915	890	784	867	890	742
*Dde*I	551, 166, 10	551, 354, 10	551, 354, 10	551, 329, 10	551, 223, 10	551, 306, 16	551, 329, 10	295, 139, 137, 125, 46
*Hin*fI	461, 174, 57, 35	649, 174, 57, 35	649, 174, 57, 35	624, 174, 57, 35	518, 174, 57, 35	601, 174, 57, 35	624, 174, 57, 35	566, 141, 35
*Tru*9I (*Mse*I)	468, 219, 31, 9	468, 215, 191, 31, 9	468, 407, 31, 9	468, 382, 31, 9	468, 276, 31, 9	468, 359, 31, 9	468, 203, 179, 31, 9	418, 266, 49, 9
*Sdu*I	319, 259, 149	319, 256, 191, 149	447, 319, 149	422, 319, 149	319, 316, 149	399, 319, 149	319, 231, 191, 149	319, 275, 148

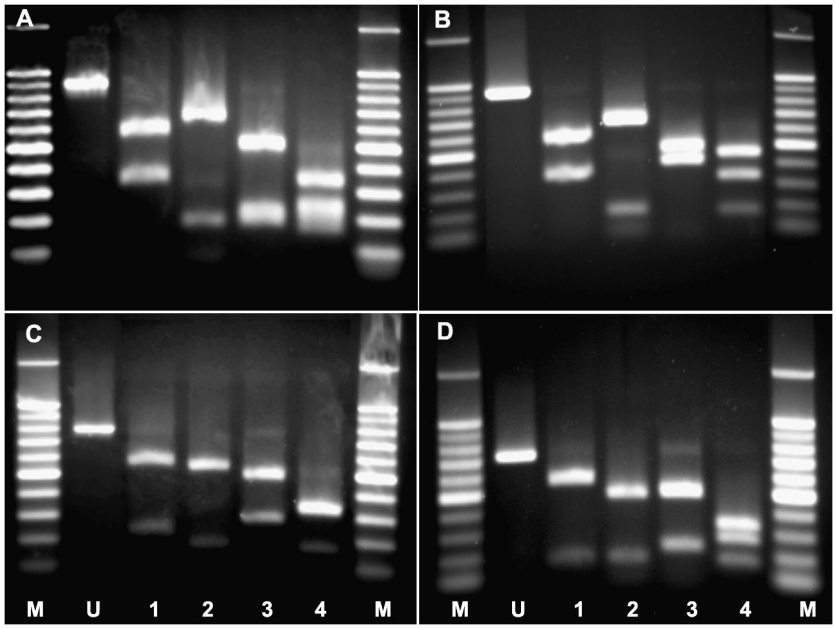

Fig. 5. *PCR-RFLP of the ITS haplotypes for* Ditylenchus destructor. *A: Haplotype G; B: Haplotype C; C: Haplotype E; D: Haplotype A. Lanes: M = 100 bp DNA ladder (Promega); U = unrestricted PCR product, 1 =* Dde*I; 2 =* Hin*fI; 3 =* Tru*11; 4 =* Sdu*I*.

(expected PCR product size = 453 bp) proposed by Marek *et al*. (2010) is also not able to detect haplotype A.

Phylogenetic relationships between *D. destructor* populations

Phylogenetic relationships between *D. destructor* populations as inferred from the analysis of the ITS1-5.8S-ITS2 rRNA gene sequences is given in Figure 7. All samples are divided into two main clades: *i*) Clade I with samples having helix H9 in ITS1 sequences (PP = 74) belonging to haplotypes B-G; and *ii*) Clade II with samples without helix H9 in ITS1 sequences (PP = 99) belonging to haplotype A. Clade I is subdivided into four subclades corresponding to three ITS haplotypes (F, B, G) and clustered together with haplotypes C, D and E (PP = 98).

The BI majority rule consensus tree reconstructed from the D2-D3 of 28S rRNA gene sequence dataset is pre-

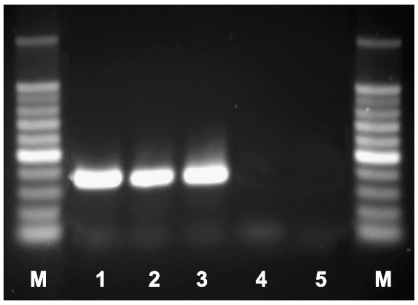

Fig. 6. *Gel with amplification products obtained in PCR with species-specific primer. Lanes: M = 100 bp DNA ladder (Promega); 1 =* D. destructor*, China, Weifang; 2 =* D. destructor*, China, Luoyang; 3 =* D. destructor*, Nizhnii Novgorod; 4 =* D. dipsaci*, onion, Russia; 5 = control without DNA.*

sented in Figure 8. Two main clades on the D2-D3 tree were observed and these seemed to correspond to two main population groups of ITS haplotypes: *i*) Clade I containing samples with helix H9 in ITS1 sequences (PP = 87); and *ii*) Clade II containing samples without helix H9 in ITS1 sequences (PP = 65). Restriction of PCR products by *Bsh*143I, *Bsh*1236I or *Rsa*I distinguished samples belonging to these clades from each other (Fig. 9). The relationships between groupings in D2-D3 and ITS trees were not verified for some samples because of the lack of ITS data.

Discussion

Previous and present studies have revealed that the ITS rRNA gene for *D. destructor* characterised by extensive length variation, which has not been previously reported for any other nematodes. The differences in this rRNA fragment length were due to the presence of repetitive elements in the ITS1. Marek *et al*. (2010) found repetitive elements of three, four and 11 nucleotides in the ITS1 of *D. destructor*; moreover, the latter one (GCATTTGT GCT) was present in two copies in studied sequences of European *D. destructor*. In our study we found that these long repetitive elements have consensus sequences: KC TRTGTRCYTGC and GCTYKYATTWGH and showed that these repetitive elements formed a stable stem structure in the ITS1 secondary structure model. Minisatellite internal repeats appear to be characteristic of the evolution of the ITS1 in several groups of organisms. Long and short repeats of ITS1 were found in mosquitoes (Paskewitz *et al.*, 1993; Bower *et al.*, 2009), trematodes (van Herwerden *et al.*, 1999; Warberg *et al.*, 2005), ladybird beetles (von der Schulenburg *et al.*, 2001), pinyon pines (Gernandt *et al.*, 2001) and in several fungus groups (Platas *et al.*, 2001; den Bakker *et al.*, 2004). Several studies showed that these repeats also formed long, stem-loop secondary structures in ITS1. Thus, this finding of repeats in *D. destructor* is the first report of minisatellites in the ITS1 for nematodes. The role of repetitive elements in the rRNA gene sequences is still unknown, although several recent reviews have highlighted the contribution of repetitive loci in adaptive evolution (Biemont & Vieira, 2006). ITS with minisatellites seems to be characteristic for several *D. destructor* population groups, which makes it a good PCR marker for population genetics and biogeographical studies of this species.

The putative ITS2 secondary structure reconstructed here is similar to those proposed for *D. dipsaci* by Subbotin *et al.* (2005) and for *D. destructor* by Marek *et al*. (2010). Slight differences in structures proposed in our study from those by Marek *et al*. (2010) can be explained by the different methods and folding parameters used and may also reflect the dynamic nature of the ITS2 structure flexing between alternative folding patterns (Ma *et al.*, 2008). The ITS1 transcript of *D. destructor* proposed in our study represents three domain structures. It is already known that three to nine ITS1 structural domains have been found in different organisms (Sun *et al.*, 2010).

Knowledge of RNA secondary structure is becoming increasingly important in assisting phylogenetic analysis through three main approaches: *i*) finding the best homology between nucleotides during alignment procedures and generation of optimal alignment; *ii*) determining the source of additional data incorporating both structural and morphometric parameters of rRNA molecules; and *iii*) selecting an increasingly appropriate model of rRNA evolution (Subbotin *et al*., 2007). Although in the present work we only improved alignment for *D. destructor* sequences using secondary structures of the ITS1, other approaches could be also applied in future studies to improve accuracy in estimation of phylogenetic relationships between potato rot nematode populations.

The observed ITS sequence divergence between studied *D. destructor* populations, at 2.6%, is, even after excluding repetitive elements with anomalous evolution, higher than intraspecific ITS sequence variations for other plant-parasitic *Ditylenchus* species which only reach 1.6% (Vovlas *et al.*, unpubl.). It allows the suggestion that *D. destructor* might represent a complex of species or subspecies. Analysis of the ITS rRNA, D2-D3 of 28S rRNA and 18S rRNA gene sequences clearly showed separation of *D. destructor* into two main groups correspond-

Fig. 7. *The 50% majority rule consensus tree from Bayesian analysis generated from the ITS-rRNA gene sequence dataset for* Ditylenchus destructor *using the GTR + I + G model. Posterior probability more than 70% is given for appropriate clades. Harmonic mean = −2822.83, arithmetic mean = −2749.84. The newly obtained sequences are indicated in bold.*

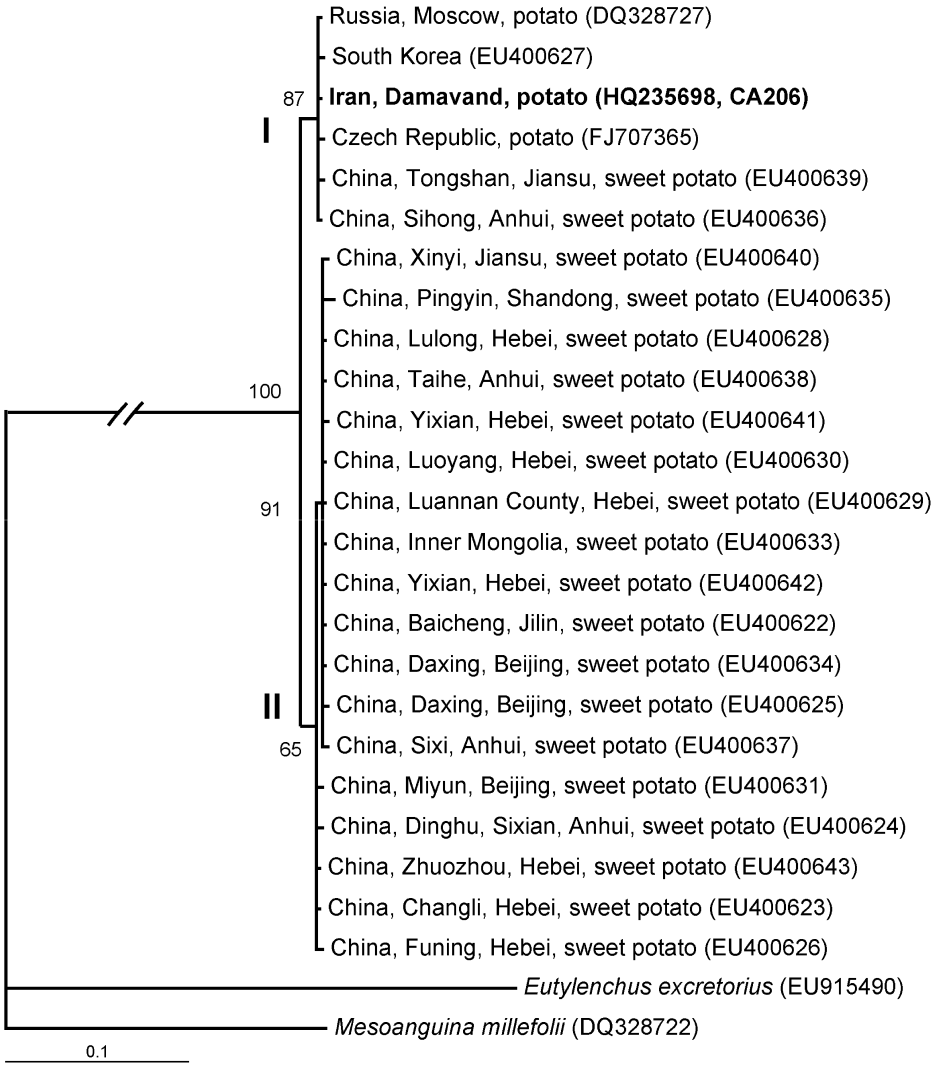

Fig. 8. *The 50% majority rule consensus tree from Bayesian analysis generated from the D2 and D3 of 28S rRNA gene sequence dataset for* Ditylenchus destructor *using the GTR + I + G model. Posterior probability of more than 70% is given for appropriate clades. Harmonic mean = −2156.56, arithmetic mean = −2118.59. The newly obtained sequence is indicated in bold.*

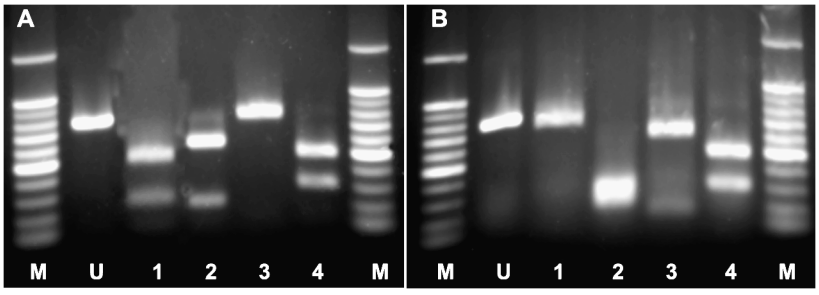

Fig. 9. *PCR-RFLP of D2-D3 of 28S rDNA for* Ditylenchus destructor. *A: RFLP profile for a population belonging to Clade I and containing helix H9 in ITS1; B: RFLP profile for a population belonging to Clade II without helix H9 in ITS1. A. Lanes: M = 100 bp DNA ladder (Promega); U = unrestricted PCR product; 1 =* Bsh*143I; 2 =* Bsh*1236I; 3 =* Rsa*I; 4 =* Hin*fI*.

ing to the ITS haplotype A and the ITS haplotypes B-G, respectively. Recent analysis of Inter Simple Sequence Repeats of *D. destructor* populations from China also showed that these molecular markers distinguished the two groups (Huang *et al.*, 2010). Moreover, after morphometric comparison, Huang *et al.* (2009) reported differences in indices c, V, V′ and tail lengths between specimens from populations belonging to the ITS haplotypes A and B. All these results indicate possible limitations in gene flow between these population groups. On the other hand, Xu *et al.* (2009) did not report any morphological or morphometric differences between specimens from populations parasitising yam, *Dioscorea opposita* Thunb. (ITS haplotype A) and *Astragalas propinquus* Schischkin (ITS haplotype F), and considered these populations as belonging to the same species. Also, there were no correlations of malate dehydrogenase phenotypes or pathogenicity grouping and the ITS haplotypes (A and B) (Wang *et al.*, 2009). Further comprehensive molecular and morphological analyses of different populations from different host plants and world regions need to be conducted in order to see if *D. destructor* is indeed a polytypic species or not.

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