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RESEARCH PAPER

Insights into the structure and phylogeny of the 28S rRNA expansion segments D2 and D3 of the plant-infecting nematodes from the genus *Ditylenchus* (Nematoda: Anguinidae)

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Summary. Recently, it has been shown that the stem nematode, *Ditylenchus dipsaci* (Nematoda: Anguinidae), is genetically more related to the gall-forming nematodes from genera *Anguina*, *Heteroanguina*, and *Mesoanguina* than to other members of the genus *Ditylenchus*. This finding was provided by molecular data written in the evolutionary variable, non-coding internal transcribed spacers (ITS1 and ITS2) of the ribosomal DNA (rDNA). In the current paper, we analyze the nucleotide sequences and predict the secondary structures of two expansion segments (D2, D3) of the 28S ribosomal RNA (rRNA)-coding gene for the plant-parasitic nematodes from the genus *Ditylenchus* and their related anguinids. In general, the expansion segment D2 appeared to be more variable than the segment D3 illustrating their different evolutionary constraints. Comparative analysis of the aligned sequences and predicted secondary structures revealed similar trend showing the tight relationships between the stem nematodes (*D. dipsaci*, *D. gigas*, *D. weischeri*) and gall-forming nematodes from the subfamily Anguininae. Phylogeny reconstructions disjoined the family Anguinidae into two monophyletic clusters (Clade 1 and 2). Clade 1 constitutes the stem nematodes (*D. dipsaci*, *D. gigas*, etc) and gall-forming nematodes from the genera *Anguina*, *Heteroanguina*, *Subanguina* and *Mesoanguina*, while clade 2 includes other *Ditylenchus* species like *D. destructor* and *D. halictus*. Collectively, deciphering the exact phylogenetic relationships within the family Anguinidae (Nematoda: Tylenchida) with respect to our results should provide a framework for a taxonomic revision in order to reflect biological history of these nematodes. In addition, we provide novel molecular data, which may be exploited in diagnostic tools for phytosanitary control of these economically important plant parasites.

Key words: D2-D3 28S rRNA, *Ditylenchus*, nematode, ribosomal DNA (rDNA), phylogeny.

Introduction

The genus *Ditylenchus* (Nematoda: Anguinidae) comprises obligate parasites with a great variety of feeding habits (Sturhan and Brzenski, 1991). Although most species of the genus are strictly mycophagous, e.g. *D. halictus* (Giblin-Davis *et al.*, 2010), there are five plant-parasitic species of high phytosanitary importance including *D. dipsaci*, *D. gigas* (Vovlas *et al.*, 2011), *D. destructor*, *D. angustus* and *D. africanus* (Brzenski, 1991; Moens and Perry, 2009).

The stem nematode, *D. dipsaci*, is the most significant agricultural and quarantine pest, especially in temperate areas of both the northern and southern hemispheres. This importance is not only the result of its wide host range covering over 500 species of angiosperms (EPPO, 2008), but also its unique ability to survive in dehydrated state, the so-called anabiosis,

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in absence of host plants (Wharton and Barrett, 1985). In addition, strong synergism between *D. dipsaci* and other pathogenic organisms, for instance some fungi (Hillnhutter *et al.*, 2011), often results in severe crop damages. The effective pest control is quite difficult because of the existence of nematode populations showing specific feeding preferences (Janssen, 1994). These populations, which are sometimes designated as races, can be distinguished by limited ranges of their host plants and partial reproduction isolation (Brzenski, 1991; Janssen, 1994). Presence of so many distinct populations (races), some of them with polyploid, led to a hypothesis about the so-called species complex *D. dipsaci* (Sturhan and Brzenski, 1991; Subbotin *et al.*, 2004; Subbotin *et al.*, 2005). Some nematologists (Sturhan and Brzenski, 1991; Subbotin *et al.*, 2005) speculate that the species *D. dipsaci* is being currently undergone speciation process, which recently resulted in designations of several new species singled out from the *D. dipsaci* species complex - *D. gigas* (Vovlas *et al.*, 2011) and *D. weischeri* (Chizhov *et al.*, 2010).

Second high-impact phytoparasitic species from the genus *Ditylenchus* is the potato rot nematode, *D. destructor* (Brzenski, 1991; Sturhan and Brzenski, 1991). This nematode especially attacks potatoes in Europe, sweet potatoes in Asia, and finally significant economic losses are also caused on groundnut-producing fields in South Africa (EPPO, 2008). In spite of *D. dipsaci*, species *D. destructor* lacks ability to survive in dehydrated state (anabiosis) for long time. So far, there are no described intra-species populations (races) with feeding preferences as in the case of *D. dipsaci*. However, our previous study (Marek *et al.*, 2010) revealed presence of repetitive genetic elements, the so-called minisatellites, in the ITS1 rDNA, which were recently used to designate seven different haplotypes within the species (Subbotin *et al.*, 2011).

At present, there are several strategies how to keep ditylenchid parasites under economic threshold that combine precise and reliable molecular diagnostics (Marek *et al.*, 2005; Subbotin *et al.*, 2005; Marek *et al.*, 2010), effective crop rotation system with perspective of cultivation of parasite-resistant transgenic crops (Gao *et al.*, 2011), quarantine precaution (EPPO, 2008), or alternatively application of nematicidal pesticides (EPPO, 2008). For this reason, studying the nematode genetic information is crucial to understand in detail all features of nematode biology. Moreover, sequence data provided by genomic studies provide a valuable source of information for molecular taxonomy, phylogenomics, and DNA-diagnostics.

Two species, *D. dipsaci* and *D. destructor*, are morphologically very similar, and it is difficult to recognize them from each other by microscopic observation (Brzenski, 1991). However, recent molecular analyses based on ribosomal DNA (rDNA) sequence data have unexpectedly provided evidence that these two species are genetically more distant than we believed before (Marek *et al.*, 2010). On the other hand, comparative analysis of the evolutionary highly variable, non-coding internal transcribed spacers (ITS1 and ITS2) of the nuclear rDNA revealed remarkable close relationships between the stem nematode, *D. dipsaci*, and the gall-forming nematodes from the subfamily Anguininae (Marek *et al.*, 2005; Subbotin *et al.*, 2005; Marek *et al.*, 2010; Vovlas *et al.*, 2011). In addition, previous phylogeny reconstructions (Subbotin *et al.*, 2006) failed to solve evolutionary boundaries between ditylenchid and anguinid taxa. Therefore, further studies are still needed to provide additional molecular data, which could help (i) to resolve phylogenetic relationships and (ii) to remove taxonomic inconsistency within the family Anguinidae.

In the current study, we determined nucleotide sequences of the D2-D3 expansion segments of the 28S large subunit (LSU) rRNA-coding gene for several populations of *D. dipsaci* and *D. destructor*. Comparative analyses of the obtained sequences and their predicted secondary structures confirmed that *D. dipsaci* is more related to the gall-forming nematodes from the genera *Anguina*, *Heteroanguina*, *Subanguina*, and *Mesoanguina* than to the other ditylenchids, particularly *D. destructor*. This taxonomic inconsistence was subsequently confirmed by molecular phylogeny reconstructions based on both the D2-D3 28S-rDNA and ITS1-5.8S-ITS2 rDNA data. Taken together, our results provide valuable information about phylogenetic relationships and molecular features of phytosanitary important nematodes from the family Anguinidae.

Materials and methods

Nematode isolates and DNA preparation

Five isolates of *D. dipsaci* and one of *D. destructor* were experimentally examined. Detailed information about the geographic origins, host preferences and sources of used nematodes are listed in Table 1.

Nematodes were eluted from infected plant tissues using the Baermann's technique as previously published (Marek *et al.*, 2010). Five nematode specimens were transferred into a sterile 1.5-mL tube containing 10 μL of sterile H_2O. Nematodes were subsequently homogenized with a hand pestle and DNA was extracted with the Qiamp DNA Mini Kit (Qiagen, Mainz, Germany) according to manufacturer's instructions.

PCR amplification

To amplify DNA regions encompassing the expansion segments D2 and D3 of the 28S-rDNA, universal primers D2A (5´-ACAAGTACCGTGAGGGAAAGTTG-3´) and D3B (5´-TCGGAAGGAACCAGCTACTA-3´) (Ellis *et al.*, 1986; Courtright *et al.*, 2000) were used. PCR reactions were performed in 0.2-mL tubes with 25 μL final volume of reaction mixture containing 2.50 μL of 10´ buffer for DNA polymerase (Fermentas, Vilnius, Lithuania), 3 μL 25 mM $MgCl_2$ (Fermentas), 0.25 μL 25mM dNTP (Fermentas), oligonucleotide primers 25 μM, and 0.40 μL of LA DNA polymerase (Fermentas) 2.5U (0.50 μL), DNA 1.00 μL and sterile H_2O 17.35 μL. The profile of gradient PCR was as follows: initial denaturation 95ºC 2 min, followed by 35 cycles (94ºC 1 min, 45ºC to 65ºC 30 s, and 72ºC 1 min) and final extension 72ºC 4 min. The generated PCR products were separated by electrophoresis in 1.0 % (w:v) agarose gel with 1´ TBE buffer, stained with ethidium bromide and visualized under UV light. The lengths of DNA fragments were estimated by comparison with MassRuler Low Range 100-bpDNA ladder or MassRuler MixDNA ladder (Fermentas).

Cloning and DNA sequencing

The amplified DNA fragments were gel-purified using the Min Elute Gel Extraction Kit (Qiagen) and cloned into the pTZ57R/T plasmid (Fermentas) as previously described (Marek *et al.*, 2005). The DNA sequencing was performed in Genomac International (The Czech Republic). Three independent clones of each nematode isolate were analysed. The consensus sequences were deposited in the GenBank database (see Table 1 for Genbank accessions).

Bioinformatics-based tools and phylogeny reconstructions

The rDNA sequences were either experimentally determined as the above described or retrieved from the non-redundant GenBank database at the National Center for Biotechnology Information (Sayers *et al.*, 2011). Multiple sequence alignments were constructed using ClustalX 2.0 (Larkin *et al.*, 2007) with a gap-opening penalty of 10 and gap-extension penalty of 0.05. Minor manual adjustments were performed in BioEdit (Hall, 1999) in order to minimize the occurrence of mis-aligned sequences. Final graphical visualization of aligned sequences was in GeneDoc (Nicholas *et al.*, 1997) and JalView (Waterhouse *et al.*, 2009). Evolutionary divergence (p-distance) between nucleotide sequences were computed from pair-wise analysis using the Maximum Composite Likelihood method implemented in MEGA4 package (Tamura *et al.*, 2007).

For phylogeny reconstructions, all columns containing gaps were excluded from multiple alignments for further analysis. The phylogenetic trees were built on the basis of multiple alignments using

Table 1. *Ditylenchus* species sequenced in this study.

***Ditylenchus* species**	**Host plant species (common name)**	**Origin**	**Source**	**GenBank accession number**
D. dipsaci	*Cichorium inthybus* (Leaf chicory)	Slovenia	G. Urek	FJ707360
D. dipsaci	*Allium sativum* (Garlic)	Czech Republic	P. Havránek	FJ707361
D. dipsaci	*Narcissus* sp. (Daffodil)	Czech Republic	V. Gaar	FJ707362
D. dipsaci	*Pisum sativum* (Pea)	USA	H. Bennypaul	FJ707363
D. dipsaci	*Medicago sativa* (Alfalfa)	Canada	H. Bennypaul	FJ707364
D. destructor	*Solanum tuberosum* (Potato)	Latvia	V. Gaar	FJ707365

the maximum likelihood (ML) method as implemented in the software package MEGA4, which was also used for bootstrap analysis (500 replicates) and graphical representation of the resulting trees.

RNA secondary structure prediction

RNA secondary structure features were analogously inferred as previously described (Marek *et al.*, 2010). Briefly, structural two-dimensional (2D) models for D2 and D3 28S rRNA expansion segments for *D. dipsaci* (GenBank accession FJ707363), *D. destructor* (FJ707365), *Heteroanguina graminophila* (DQ328720), *Anguina tritici* (DQ328723) and *Subanguina radicicola* (DQ328721) were computed by the Mfold algorithm (Zuker, 2003). Screening for thermodynamically optimal and suboptimal secondary models was performed with help of the RNAstructure 4.4 software package (Mathews, 2006). The default folding parameters were used with exception of temperature adjustment to 25°C. The predicted models were exported from RNAstructure 4.4 in ct format for final refinements and visualization with RNAViz (De Rijk and De Wachter, 1997) and XRNA (Weiser and Noller, 1995) software packages.

Results

Sequence analysis of D2-D3 expansion segments of 28S rDNA gene

The PCR amplification using D2A and D3B primers resulted in a single DNA amplicon of ~780-bp for all analyzed *Ditylenchus* species (Figure 1). The

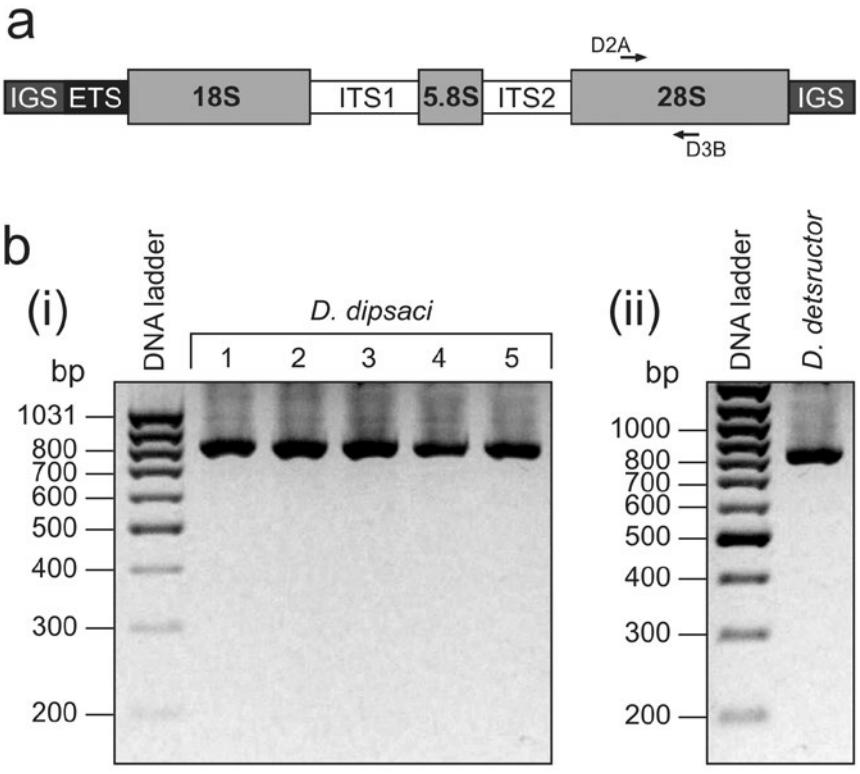

Figure 1. Amplification of D2D3 expansion segments of the 28S-LSU rRNA gene. **a.** Schematic representation of the nuclear ribosomal DNA array. PCR primer-representing arrowheads indicate amplified D2D3 expansion segments of the 28S rRNA gene. IGS, intergenic spacer; ETS, external transcribed spacer; ITS, internal transcribed spacer; 18S, 5.8S and 28S rRNA genes. **b.** Agarose gels of D2-D3 28S rDNA amplicons for *Ditylenchus dipsaci* (i) and *D. destructor* (ii). Lanes: 1, *D. dipsaci* isolate from *Cichorium inthybus*; 2, *D. dipsaci* isolate from *Allium sativum*; 3, *D. dipsaci* isolate from *Narcissus* sp.; 4, *D. dipsaci* isolate from *Pisum sativum*; 5, *D. dipsaci* isolate from *Medicago sativa*. MassRuler MixDNA ladder (Fermentas, Lithuania).

highest yield and purity of the PCR-amplified products were achieved using annealing temperature of 53.5°C, as determined by gradient-temperature PCR screening (data not shown). DNA sequencing revealed that exact sizes of the amplified D2-D3 LSU segments were 785 bp for most *D. dipsaci* isolates with exception of two *D. dipsaci* specimens isolated from narcissus and pea as plant hosts (both 784 bp). On the other hand, a 779-bp fragment was amplified for *D. destructor* species (Figure 1). The nucleotide compositions of the amplified D2-D3 segments were the following: 23.4% A, 20.8% C, 31.6% G, and 24.2% T for *D. dipsaci* and 22.2% A, 21.3% C, 31.6% G, and 24.9% T for *D. destructor*. The D2-D3 LSU segments showed ~52% GC content for both *D. dipsaci* and *D. destructor* species.

Sequence comparison of the D2-D3 LSU segments only revealed several single-point mutations (substitutions) and one deletion event, demonstrating minimal variability between *D. dipsaci* isolates (Figure 2a and Figure 3a). The highest genetic distance has been recorded for *D. dipsaci* isolated from pea (Genbank accession FJ707364). Interestingly, this genetically distant *D. dipsaci* isolate obviously shares a number of autapomorphic features with members of the subfamily Anguininae, especially with *A. tritici* (see Figure 2a). By contrast, sequence comparisons of *D. dipsaci* and *D. destructor* populations showed a high genetic distance between these two phytopathologically-relevant nematode species (Figure 2a and Figure 3a). Based on the D2-D3 LSU-rDNA alignment, it is apparently evident that *D. dipsaci* species is more related to the gall-forming nematodes from the subfamily Anguininae (e.g. genera *Anguina*, *Subanguina*, *Heteroanguina* etc) than to members of the genus *Ditylenchus*, such as *D. destructor* and *D. halictus* species (Figure 2a and Figure 3a). There are many sequence features (substitutions, deletions/insertions) discriminating between *D. destructor* on the one side and *D. dipsaci* and other anguinid taxa on the other.

This phenomenon was also confirmed by pairwise distance (p-distance) matrix calculations (Figure 2b and Figure 3b). For instance, p-distance expressing genetic distance between *D. dipsaci* and the gall-forming nematodes of the genera *Anguina*, *Subanguina*, *Heteroanguina* were in range 0.093-0.173, whilst p-distance value between *D. dipsaci* and *D. destructor* was significantly higher (0.630). All calculated p-distance values are summarized in Figure 2b and Figure 3b. In general, the D2 segment showed significantly higher sequence variability than the D3 segments, indicating their different evolutionary constrains. Taken together, the D2-D3 sequence inspections indicate tight relationships between the stem nematodes (*D. dipsaci, D. gigas*) and the gall-forming nematodes (genera *Anguina*, *Subanguina*, *Heteroanguina*, etc).

Secondary structure models for D2 segment of 28S rRNA

Using energy-minimization approach, a set of secondary structure models of the D2 and D3 expansion segments LSU-rRNA were inferred for *D. dipsaci*, *D. destructor*, *H. graminophila*, *A. tritici*, and *S. radicicola*, and these models are proposed in Figure 4. At first sight, a general secondary structure model consisting of a closed, multi-branch loop with three paired helices could be deduced for all anguinid taxa. These helical motifs (domains) are designated as Helix-1, Helix-2 and Helix-3, and subcomponents of the compound helices are named a, b, c, etc. (Figure 4). Numerical and statistical characteristics of the proposed structures are given in Table 2. The size of the closed loop lay within a frame of 7 to 15 unpaired nucleotides (nt), where *D. destructor* has the most bulky loop (15 nt). Proportions of G-U pairs in the helical domains were usually low (13.8–15.2%). The helical domains showed distinct size classes. For all proposed structures, the Helix-1 is the shortest (13–14 bp long) and forming a stem helix closing the multi-branch loop. The Helix-2 varies from 37 to 40 bp, and is ended by a conserved uridine (U)-rich loop with exception of *D. destructor* where this U-loop is strongly reduced. The Helix-3 is the longest of the three helical domains, ranging from 75 to 79 bp. The Helix-3 is typically separated into two helical subdomain. Within the Helix-3, two conserved U-U mismatches can be found with exception of *D. destructor* D2-LSU rRNA where only one U-U mismatch is formed (Figure 4). Careful inspections revealed a conserved core of the D2-LSU rRNA structure shared by all anguinid taxa. This core is represented by the central multi-branch loop with flanking Helix-1 and basal regions of the Helix-2 and Helix-3 (see Figure 4). Importantly, *D. dipsaci* D2-LSU rRNA structure markedly resembles the proposed structure of *H. graminophila* (Figure 4). The data collected so far demonstrate the close relationships between *D. dipsaci* and the gall-forming nematodes also at the secondary structure level.

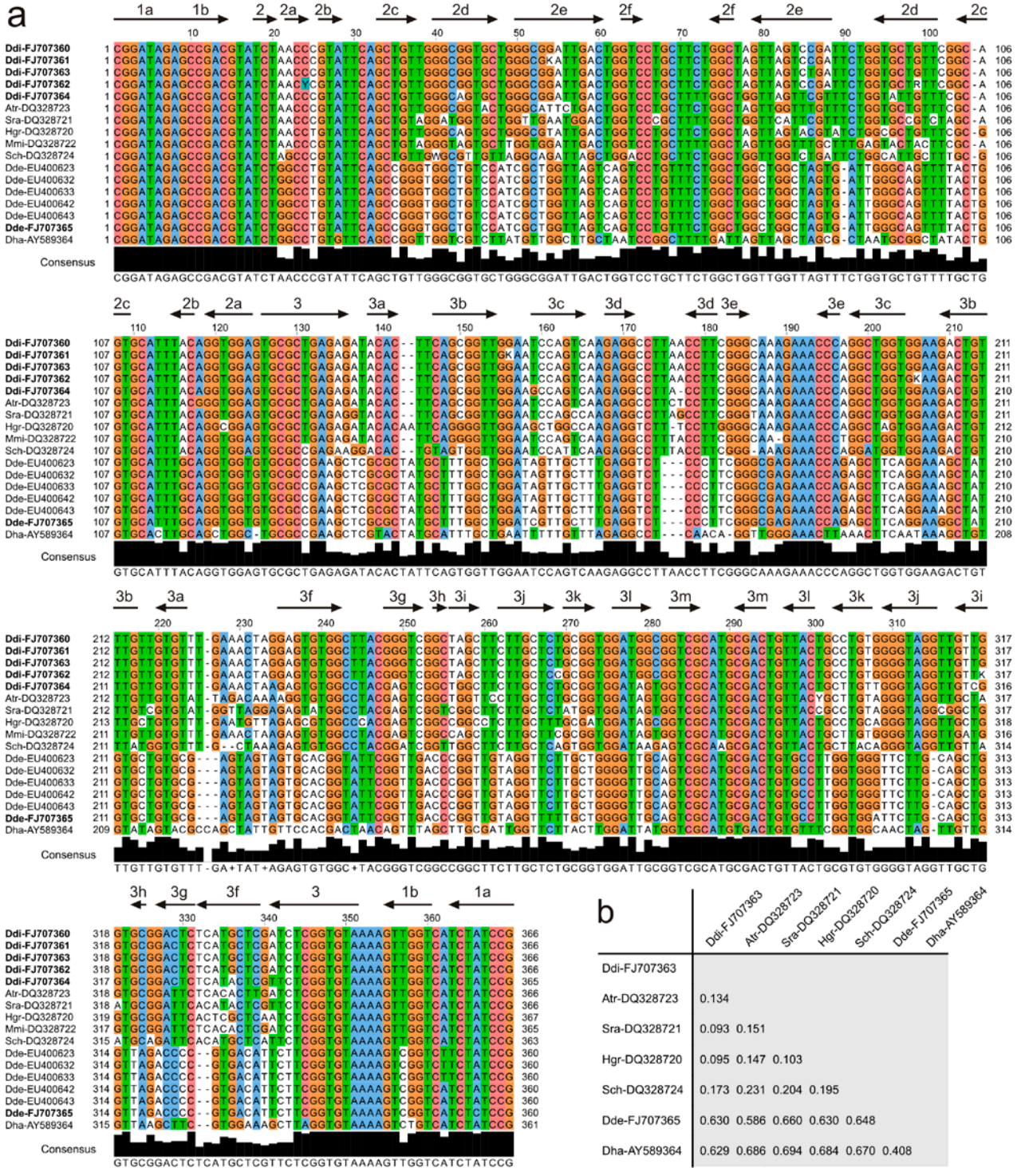

Figure 2. a. Multiple sequence alignment of the expansion segment D2 LSU 28S nuclear rRNA gene from anguinid taxa. The label on the left-hand side of each sequence gives its study code with GenBank accession. The consensus row at the bottom shows the most frequent residue at each row or "+" if two or more residues are equally abundant. Predicted secondary structural elements (helical regions) for the stem nematode *Ditylenchus dipsaci* are indicated by black arrows (→ or ←) reported above the sequences; the numbering of helices is the same as in Figure 5a. **b.** Distance matrix based on the D2-28S rDNA alignment for the selected members of the Anguinidae family. Note: For each matrix, the values are pair-wise p-distances, which represent the divergence of all bases between two sequences. Nematode species abbreviations: Ddi, *D. dipsaci*; Dde, *D. destructor*; Dha, *D. halictus*; Atr, *Anguina tritici*; Sra, *Subanguina radicicola*; Hgr, *Heteroanguina graminophila*; Mmi, *Mesoanguina millefolii*. The number following species abbreviation at each nucleotide sequence represents GenBank accession number.

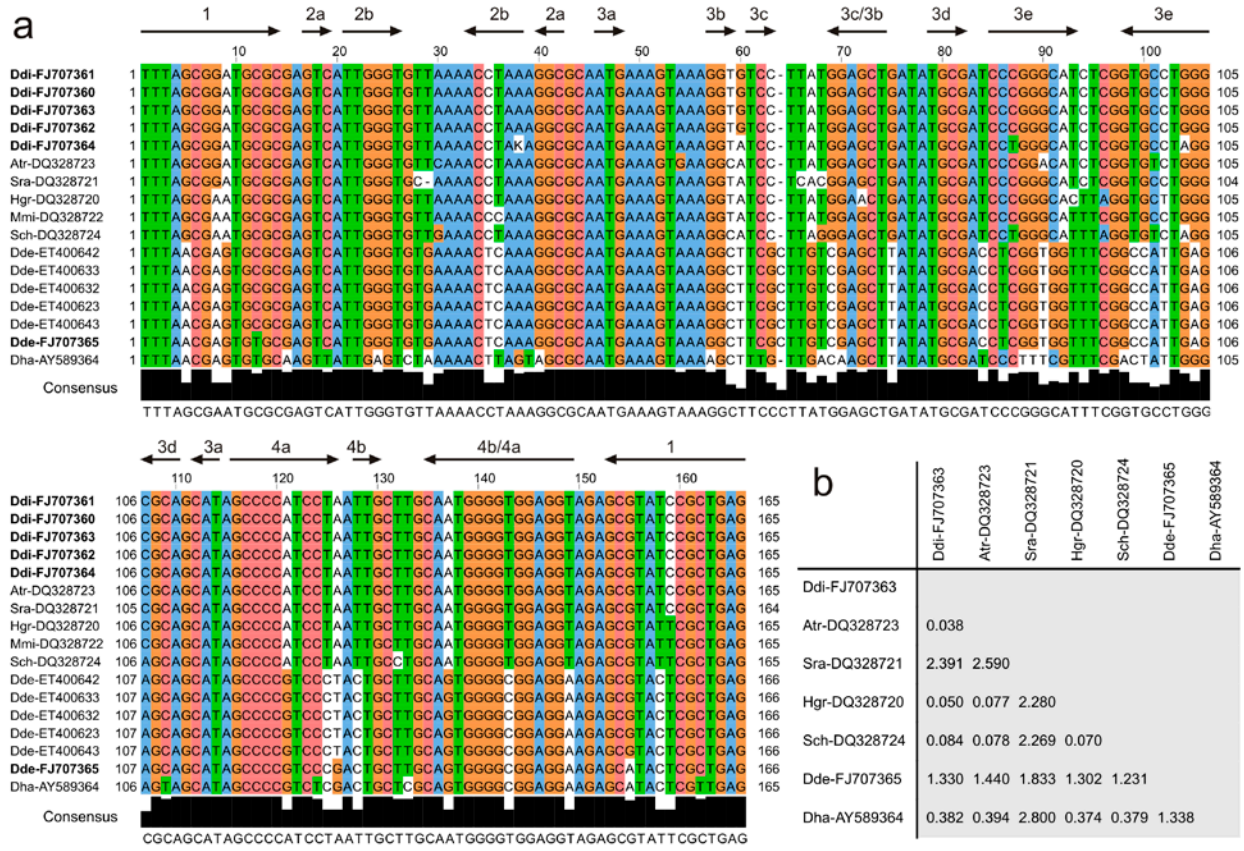

Figure 3. a. Multiple sequence alignment of the D3 LSU 28S rDNA sequences. The label on the left-hand side of each sequence gives its study code with GenBank accession. The consensus row at the bottom shows the most frequent residue at each row or "+" if two or more residues are equally abundant. Predicted secondary structural elements (helical regions) for the stem nematode *Ditylenchus dipsaci* are indicated by black arrows (→ or ←) reported above the sequences; the numbering of helices is the same as in Figure 5a. **b.** Distance matrix based on the D3-28S rDNA alignment for the selected members of the Anguinidae family. Note: For each matrix, the values are pair-wise p-distances, which represent the divergence of all bases between two sequences. Nematode species abbreviations are as in Figure 2. The number following species abbreviation at each nucleotide sequence represents GenBank accession number.

The expansion segment D3 of the LSU 28S rRNA

Computational approaches have also been applied to infer secondary structures of the D3 expansion segment of LSU-rRNA for selected anguinid taxa. The 28S-D3 region folds into a multi-branch loop, and contains four compound helices (Helix-1, Helix-2, etc) with exception of *D. destructor* where five compound helices were predicted. Analogously, subcomponents of the compound helices are named a, b, c, etc. A gallery of the proposed 28S-D3 structures is presented in Figure 5, and structural statistics are summarized in Table 3. The size of the loop lay within a frame of 9 to 25 nt, where *S. radicicola* has the most bulky loop (25 nt). Proportions of G-U pairs in the helical domains were similarly low (9.0-11.4%). The Helix-1 is closing the loop and together with the Helix-2 is highly conserved in all analyzed anguinid species. The D3-28S structural topology of *D. dipsaci* is almost identical with the structures of *A. tritici* and *H. graminophila* illustrating their shared evolutionary history. In *S. radicicola* only three compound helices can be found putatively due to mutual fusion of the Helices 3 and 4. More interestingly, the D3-28S of *D. destructor* exhibits even five compound helices (designated 1-5), showing its distance from the above described anguinid taxa.

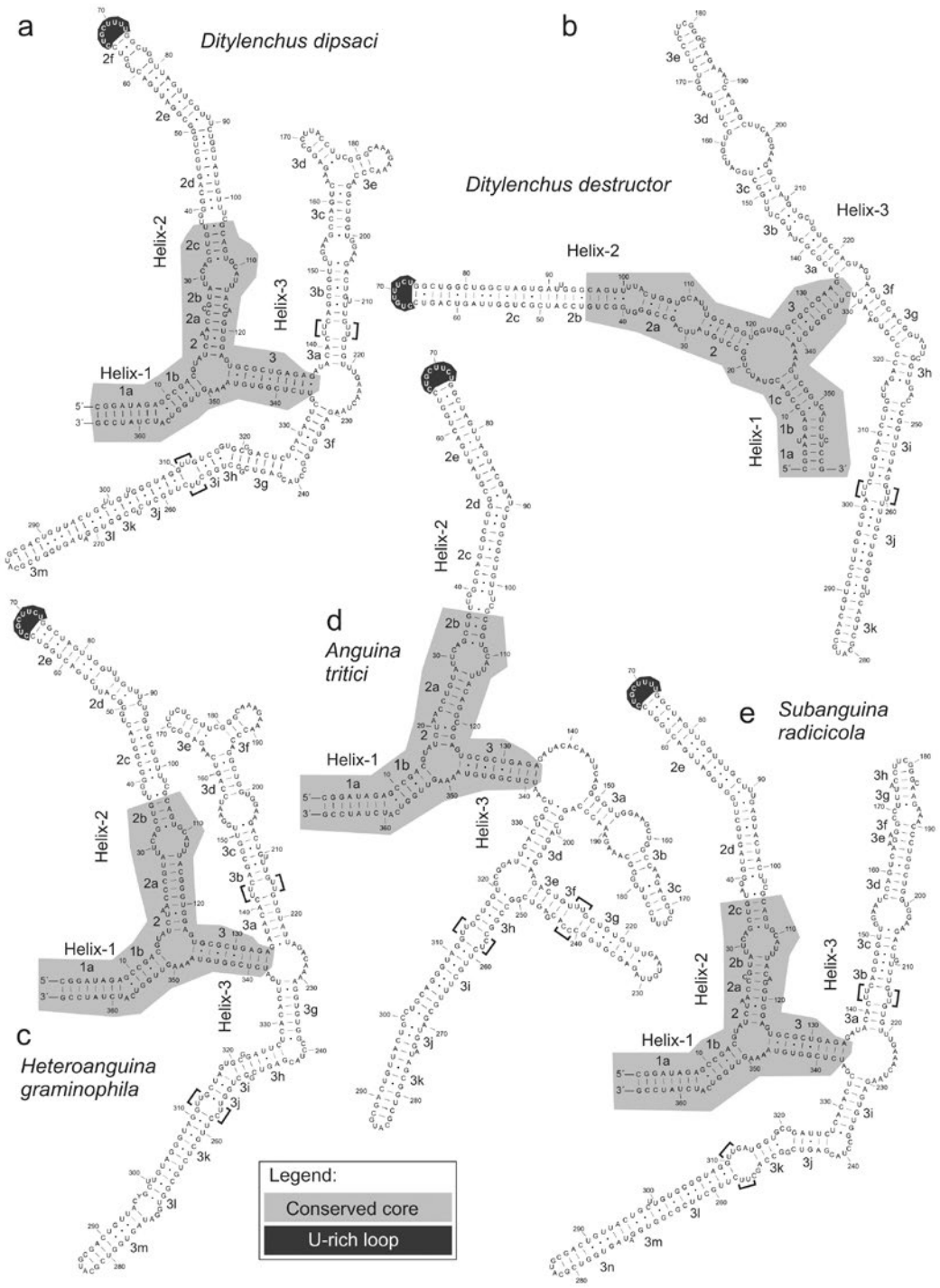

Figure 4. Secondary structure models of the D2-28S rRNA expansion segments for *Ditylenchus dipsaci* (**a**), *D. destructor* (**b**), *Heteroanguina graminophila* (**c**), *Anguina tritici* (**d**) and *Subanguina radicicola* (**e**). The models were computed by an energy-minimatization approach using the Mfold program and final adjustments of folded structures were performed with the RNAViz software. Bonds between C:G and U:A nucleotides are indicated by a straight line; bonds between U:G are indicated by a closed circle. The conserved core is highlighted in light gray field, the U-rich loop is labeled by dark grey field, and the pyrimidine mismatches are marked with square brackets.

Table 2. Statistics of the secondary structures (D2-LSU rRNA) proposed in this study.

Nematode species	Sequence length (nt)	Loop length (unpaired bases)	GC content	Paired G-U in domains I-V	Length of helices (in paired bases)			DG (25°C, kcal/mol)
					1	2	3	
Ditylenchus dipsaci	365	101	52.1%	15.2%	14	38	78	-151.1
D. destructor	360	99	55.3%	13.8%	13	38	79	-178.6
Anguina tritici	367	109	54.0%	14.7%	14	40	75	-160.0
Heteroanguina graminophila	366	108	51.9%	14.3%	14	37	79	-153.0
Subanguina radicicola	365	101	51.0%	15.2%	14	40	78	-148.7

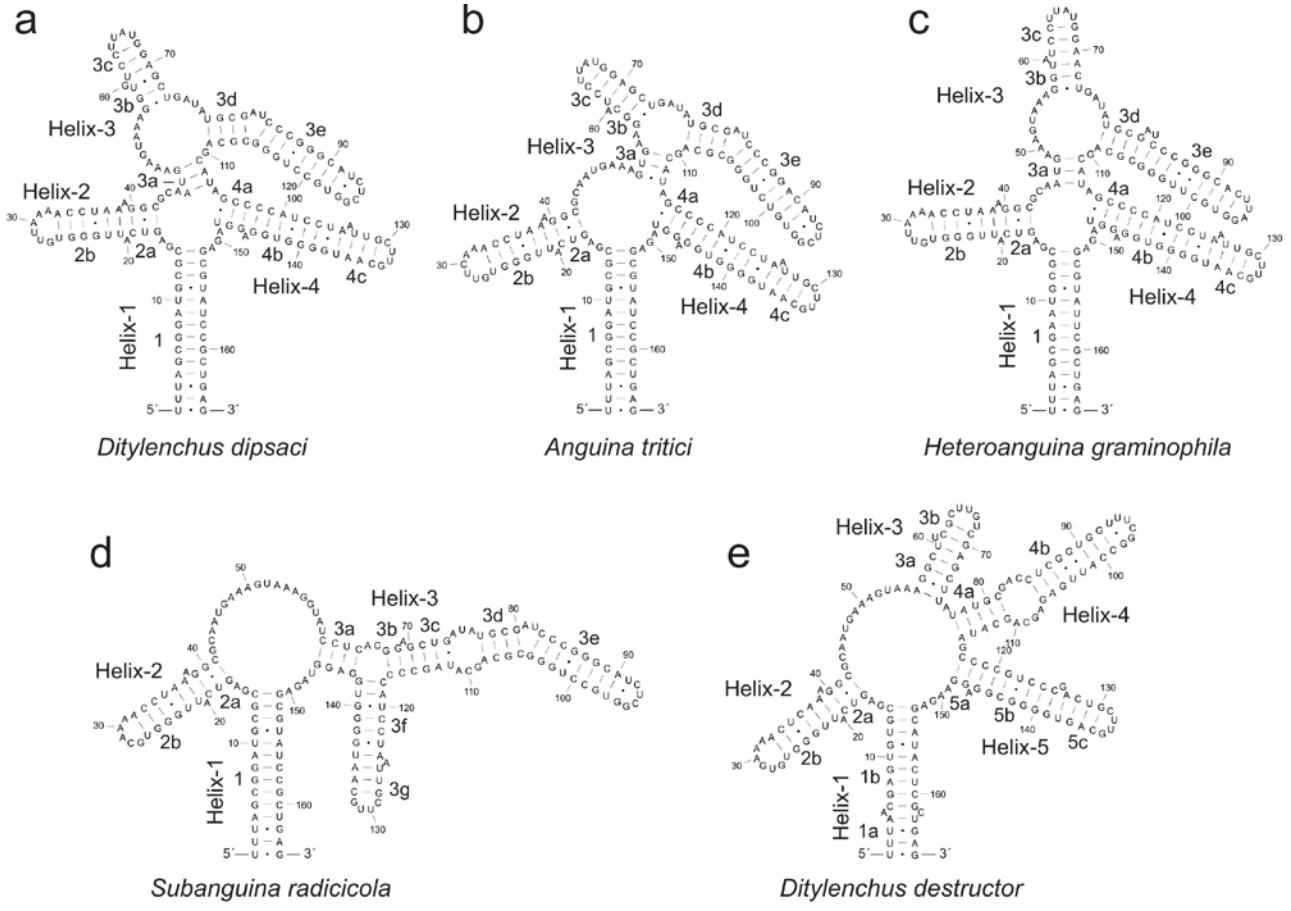

Figure 5. Secondary structure models of the D3-28S rRNA expansion segments for *Ditylenchus dipsaci* (**a**), *D. destructor* (**b**), *Heteroanguina graminophila* (**c**), *Anguina tritici* (**d**) and *Subanguina radicicola* (**e**). The models were computed by an energy-minimatization approach using the Mfold program and final adjustments of folded structures were performed with the RNAViz software. Bonds between C:G and U:A nucleotides are indicated by a straight line; bonds between U:G are indicated by a closed circle.

Table 3. Statistics of the secondary structures (D3-LSU rRNA) proposed in this study.

Nematode species	Sequence length (nt)	Loop length (unpaired bases)	GC content	Paired G-U in domains I-V	Length of helices (in paired bases)					DG (25°C, kcal/mol)
					I	II	III	IV	V	
Ditylenchus dipsaci	165	46	53.3%	10.1%	14	9	22	14	-	-72.1
D. destructor	166	52	53.6%	11.4%	13	9	7	14	13	-66.9
Anguina tritici	165	47	53.3%	10.2%	14	9	22	14	-	-74.2
Heteroanguina graminophila	165	47	49.7%	9.3%	14	9	22	14	-	-69.2
Subanguina radicicola	164	53	54.9%	9.0%	14	9	33	-	-	-71.3

Phylogeny reconstruction based on D2-D3 LSU-rDNA

To better understand relationships within the family Anguinidae, we inferred a phylogentic tree based on D2-D3 LSU sequence data (Figure 6). The tree shows overall position of the family Anguinidae within the order Tylenchida, consistent with previously published data (Subbotin *et al*., 2006). The anguinid nematodes represent a sister taxon to the group of nematodes from families Tylenchidae, Telotylenchidae, Psilenchidae, Sphaerulariidae, and Atylenchidae (Figure 6). Surprisingly, the sequence of *Neotylenchus* sp. (family Neotylenchidae) is affiliated to the anguinid clade (bootstrap value 70), suggesting their common long past evolutionary history.

Using our molecular phylogeny approach, two clades (1 and 2) were resolved within the anguinid group (Figure 6). The clade 1 comprises the stem nematode species (*D. dipsaci* and *D. gigas*), the gall-forming nematode species (*S. radicicola*, *A. tritici*, *H. graminophila*, *M. millefolii*, and *S. chilenchis*), and the above mentioned *Neotylenchus* sp. On the other hand, the clade 2 includes *D. destructor* and *D. halicactus*. It is important to note that *D. destructor* isolated from potato (*Solanum tuberosum*) tubers (Genbank accession FJ707365) was significantly separated from other populations of *D. destructor* included in the study, all isolated from sweet potatoes (*Ipomoea batatas*).

Taken together, molecular phylogeny reconstruction based on the slowly evolving 28S-rDNA gene provide evidence that species *D. dipsaci* and *D. gigas* (the stem nematodes) are more related to the group of gall-forming nematode genera (*Anguina*, *Subanguina*, *Heteroanguina*, *Mesoanguina*) than to other species of the genus *Ditylenchus*, particularly *D. destructor* (the potato rot tuber nematode). The obtained data thus suggest a taxonomic revision of the family Anguinidae with a respect to currently available molecular data.

Fast evolving ITS-rDNA regions support the taxonomic inconsistency within the family Anguinidae

To verify our findings, we also constructed phylogenetic tree of the family Anguinidae inferred from fast evolved ITS spacer segments of rDNA (Figure 7). Similar tree topology has been shown. The stem nematodes (*D. dipsaci*, *D. gigas*, and *D. weischeri*) are again closely associated with the gall-forming nematode genera (*Anguina*, *Subanguina*, *Heteroanguina*, *Mesoanguina*), while the potato tuber rot nematode (*D. destructor*) occupies sister clade together with *D. halictus* and *D. myceliophagus*. The ITS-rDNA-based findings are thus confirming the taxonomic inconsistency within the family Anguinidae that should be taken in account for the taxonomic revision.

Discussion

In the current paper, we studied sequence and secondary structure features of the expansion segments D2 and D3 of the nuclear 28S rRNA-coding gene for the plant endoparasitic nematodes from the genus *Ditylenchus*. We also addressed the question of whether molecular data contained in the D2-D3 28S rDNA confirm, or conversely falsify previously revealed genetic relationships between nematode species from the family Anguinidae (Marek *et al*., 2010).

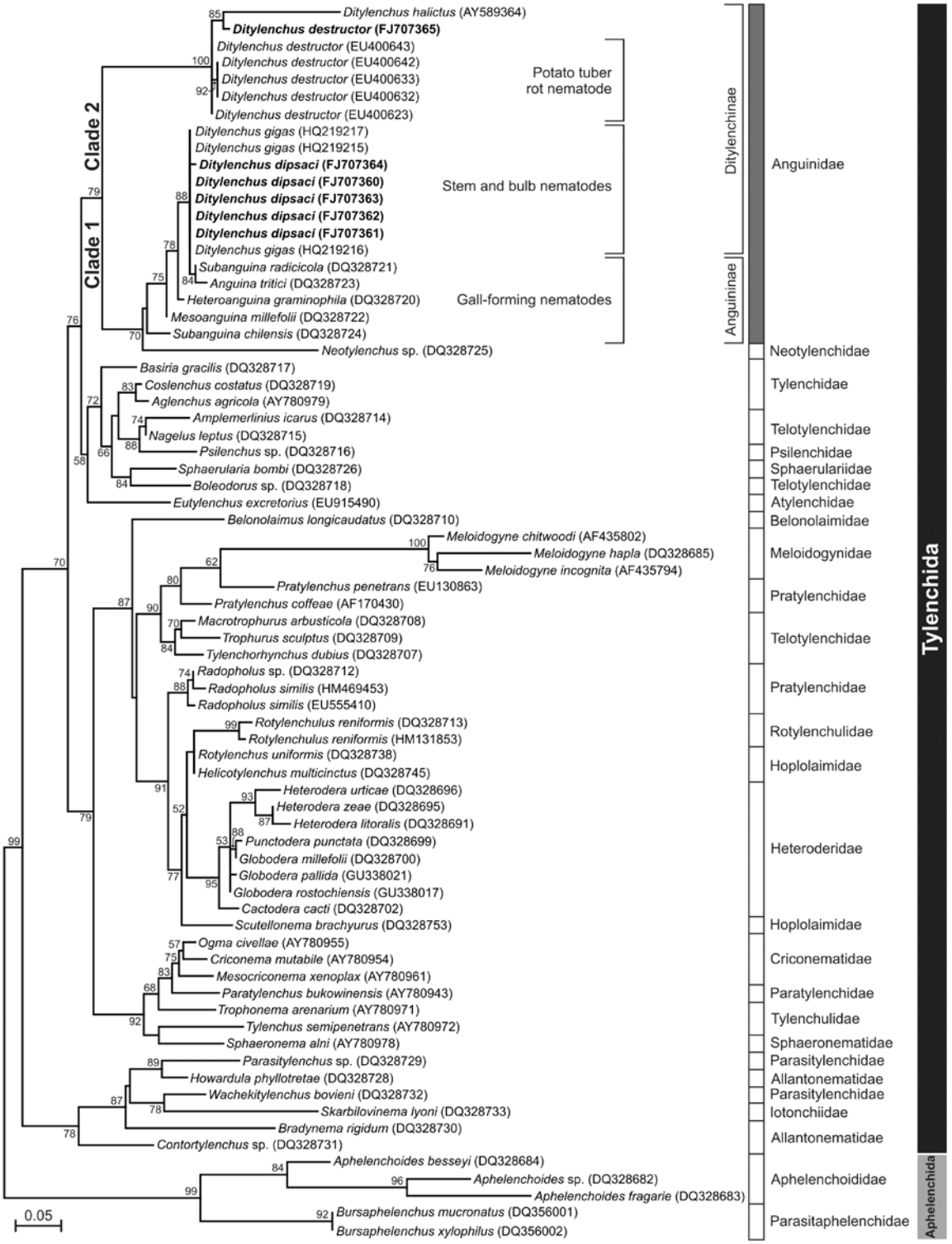

Figure 6. Unrooted maximum likelihood tree inferred from the D2-D3 LSU-rDNA sequence data for selected nematode species of the orders Tylenchida and Aphelenchida. Values at branches denote percentual bootstrap values (out of 500 replicates). The numbers in brackets indicate the GenBank accession. Newly sequenced species are indicated in bold font.

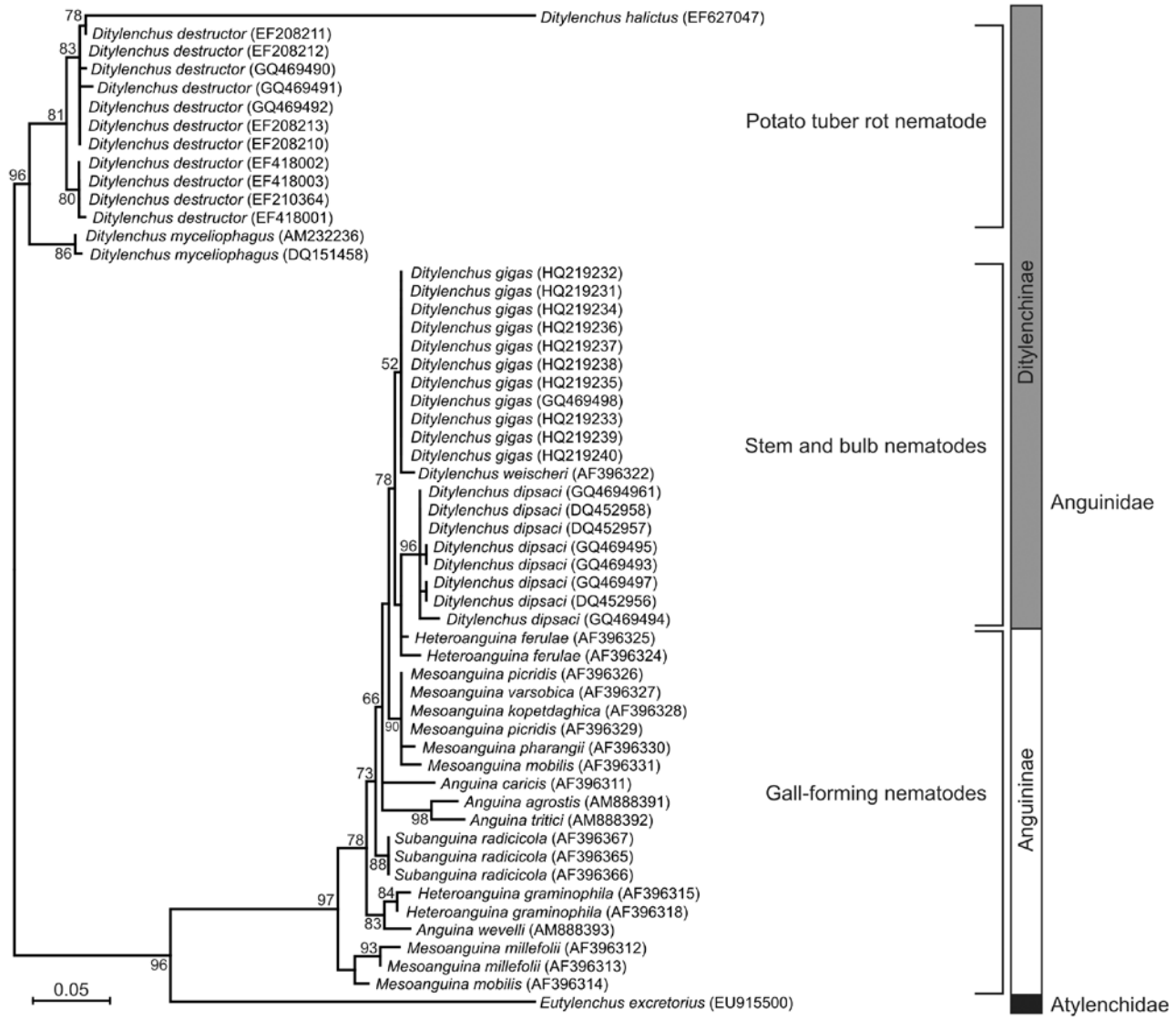

Figure 7. Maximum likelihood tree built up from the ITS1-5.8S-ITS2 rDNA sequence data. Fifty-four ITS-rDNA nucleotide sequences of anguinid species were aligned with help of ClustalX. All positions containing gaps and missing data were eliminated from the dataset, and phylogenetic analysis was conducted in MEGA4. Sequence of *Eutylenchus excretorius* (Tylenchida: Atylenchidae) was used as outgroup. Numbers at branches denote percentual bootstrap values (out of 500 replicates), and numbers in the brackets indicate GenBank accession.

To explore these issues, we determined nucleotide sequences of the D2-D3 28S rDNA for several isolates of *D. dipsaci* and *D. destructor*, and compared them with retrieved homologous anguinids sequences from the GenBank database (Figure 2 and Figure 3). Sequence comparisons definitively confirmed the close genetic relationship between the stem nematode *D. dipsaci* (subfamily Ditylenchinae) and the group of gall-forming nematodes from genera *Anguina*, *Heteroanguina*, *Mesoanguina*, and *Subanguina* (subfamily Anguininae). Conversely, sequence data contained in the D2-D3 28S rRNA-coding regions similarly showed the fact illustrating relative high genetic distance between *D. dipsaci* and *D. destructor*,

two important ditylenchid species causing economic losses on agricultural crops in temperate zone.

In the next step, we performed *in silico* reconstruction of secondary structures of both the D2 and D3 expansion segments (Figure 4 and Figure 5). In general, the predicted secondary structures of the D2/D3 segments of *D. dipsaci*, *D. destructor* and other members of the family Anguinidae possess common topologies like other eukaryotes (Gillespie *et al.*, 2004; Gillespie *et al.*, 2005; Bae *et al.*, 2010). Some minor differences may be explained by either different software defaults and/or presence of sequence differences. Importantly, the inferred structure topologies demonstrate again the close relationships between *D. dipsaci* and the gall-forming nematodes. On the other hand, *D. destructor* species, especially its D3-28S rRNA, shows the considerable distance from *D. dipsaci* and other anguinid taxa as demonstrated in Figure 4 and Figure 5.

Altogether, we provide additional molecular evidence that two endoparasite nematodes from the genus *Ditylenchus*, namely *D. dipsaci* and *D. destructor*, are more genetically distant than previously believed. Until 1945, both *D. dipsaci* and *D. destructor* were considered as a single species (see references Brzenski, 1991; Sturhan and Brzenski, 1991 for reviews). However, due to contemporary molecular tools we (Marek *et al.*, 2010) and others (Subbotin *et al.*, 2004; Subbotin *et al.*, 2005; Subbotin *et al.*, 2006) reveal more and more features distinguishing these two species. This phenomenon is strongly supported by our phylogenetic reconstruction (Figure 6 and Figure 7), where the apparent clustering of the stem nematode species (*D. dipsaci*, *D. gigas* and *D. weischeri*) and the gall-forming nematode genera (*Anguina*, *Heteroanguina*, *Mesoanguina*, and *Subanguina*) can be observed. In despite, *D. destructor* and some other ditylenchid like *D. halictus* and *D. myceliophagus* represent a monophyletic, well-defined sister clade (Figure 6 and Figure 7).

In addition to the molecular data, there are also recent morphological observations revealing some new structural differences between *D. dipsaci* and *D. destructor* (Karssen and Willemsen, 2010). Additionally, living and feeding strategies of *D. dipsaci* and *D. destructor* obviously show less common features than e.g. *D. dipsaci* and *A. tritici*. The most significant feature discriminating these two species is an inability of *D. destructor* to survive in the so-called anabiosis. On the other hand, *D. dipsaci* share this feature with other anguinids like *A. tritici*, which also indirectly demonstrates their close relationship. Hence, these findings suggest a common ancestor of current species of the stem nematodes (*D. dipsaci*, *D. gigas* and *D. weischeri*) and gall-forming nematode genera (*Anguina*, *Heteroanguina*, *Mesoanguina*, and *Subanguina*), while *D. destructor* and *D. halictus* represent their sister clade.

Lastly, deciphering the exact phylogenetic relationships within the family Anguinidae (Nematoda: Tylenchida) with respect to our results should provide a framework for a taxonomic revision in order to reflect biological history of these nematodes.

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