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ORIGINAL ARTICLE

Occurrence of *Ditylenchus destructor* Thorne, 1945 on a sand dune of the Baltic Sea

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Abstract

Ditylenchus destructor is a serious pest of numerous economically important plants worldwide. The population of this nematode species was isolated from the root zone of *Ammophila arenaria* on a Baltic Sea sand dune. This population's morphological and morphometrical characteristics corresponded to *D. destructor* data provided so far, except for the stylet knobs' height (2.1–2.9 *vs* 1.3–1.8) and their arrangement (laterally *vs* slightly posteriorly sloping), the length of a hyaline part on the tail end (0.8–1.8 *vs* 1–2.9), the pharyngeal gland arrangement in relation to the intestine (dorsal or ventral *vs* dorsal, ventral or lateral) and the appearance of vulval lips (smooth *vs* annulated). Ribosomal DNA sequence analysis confirmed the identity of *D. destructor* from a coastal dune.

Keywords: *Ammophila arenaria*, internal transcribed spacer (ITS), potato rot nematode, 18S, 28S rDNA

Introduction

Nematodes from the genus *Ditylenchus* Filipjev, 1936, are found in soil, in the root zone of arable and wild-growing plants, and occasionally in the tissues of underground or aboveground parts (Brzeski 1998). This genus includes approximately 80–90 accepted species (Brzeski 1991; Qiao *et al.* 2016) of which more than 20 have been reported in Poland (Brzeski 1998; Winiszewska 2008; Jeszke *et al.* 2014; Skwiercz *et al.* 2017). The majority of *Ditylenchus* are associated with terrestrial plants and only two representatives of this genus were found to be parasites of aquatic ones (Skwiercz *et al.* 2017). The researchers' interest in the genus *Ditylenchus* is primarily due to the role which some of the representative species play in agriculture. An intensive growth of the populations of these species in soil may lead to wilting and death of their host plants, causing decreased or even loss of yields (Sturhan and Brzeski 1991; Fourie *et al.* 2017).

Economically important species include: *Ditylenchus africanus* Wendt *et al.*, 1995 and *Ditylenchus arachis* Zhang *et al.*, 2014, both of which are pests of peanut (*Arachis hypogaea* L.), *Ditylenchus destructor* Thorne, 1945 which feeds on potato (*Solanum tuberosum* ssp. *tuberosum* L.) and numerous ornamental plant species, *Ditylenchus dipsaci* (Kühn, 1857) which is an important pest primarily of onion (*Alium cepa* L.), *Ditylenchus gigas* Vovlas *et al.*, 2011 which feeds on broad bean (*Vicia faba* L.) and *Ditylenchus myceliophagus* Goodey, 1959, which destroys the mycelium of mushroom (Kühn 1857; Thorne 1945; Goodey 1959; Wendt *et al.* 1995; Vovlas *et al.* 2011; Zhang *et al.* 2014). For other *Ditylenchus* species associated with higher plants, no negative impact on the growth and development of their host plants was observed.

Due to their specificity, coastal dunes are an interesting research environment, both for ecologists and zoologists (Kisiel 1966; Arens *et al.* 2001; Brinkman *et al.* 2004, 2005; van der Putten *et al.* 2005; van der Stoel and van der Putten 2006; Speybroeck *et al.* 2008; Wall *et al.* 2008; Mateille *et al.* 2014; Tzortzakakis

et al. 2016). From the root zone of plants growing on sand dunes, many new nematode species, including plant-parasitic nematodes have been described. These include: *Pratylenchoides arenarius* Brzeski, 1998, *Pratylenchus brzeskii* Karssen *et al.*, 2000, *Longidorus balticus* Brzeski *et al.*, 2000, *Longidorus dunensis* Brinkman *et al.*, 1987, *Meloidogyne duytsi* Karssen *et al.*, 1998, *M. maritima* (Jepson, 1987), *M. dunensis* Palomares-Rius *et al.*, 2007 and *Heterodera arenaria* Cooper, 1955 (Cooper 1955; Brinkman *et al.* 1987; Jepson 1987; Brzeski 1998; Karssen *et al.* 1998; Karssen *et al.* 2000; Brzeski *et al.* 2000; Palomares-Rius *et al.* 2007).

In this environment, mainly in the root zone of the grass *Ammophila arenaria* (L.) and *Elymus arenarius* L., representatives of the genus *Ditylenchus* were also found, including: *Ditylenchus anchilispossomus* Fortuner, 1982, *D. kheiri* Fortuner *et* Maggenti, 1987, *D. dipsaci*, *D. myceliophagus*, *D. oncogenus* Vovlas *et al.*, 2015 and *D. valveus* Thorne *et* Małek, 1968 (Thorne and Małek 1968; Kisiel 1970; Fortuner 1982; Fortuner and Maggenti 1987; Knevel 2001; Verschoor 2001; Schreck Reis *et al.* 2005; van der Putten *et al.* 2005; Mateille *et al.* 2011; Mateille *et al.* 2014; Vovlas *et al.* 2015) (Brzeski-collection of MiZ PAN Warsaw preparations).

As part of research carried out in the area of the white Baltic dunes on nematodes from the genus *Ditylenchus* in Poland, a population of nematodes morphologically similar to *D. destructor* was found for the first time in the root zone of European marram grass (*A. arenaria*). A detailed study of the population's morphology and morphometry was conducted and its 18S, 28S and internal transcribed spacer (ITS) ribosomal DNA partial sequences were acquired and analysed. The obtained results were compared with additional data from populations of *D. destructor* feeding on potatoes, collected in Lubelskie and Łódzkie Voivodeships in Poland. This article presents the results of the research.

Materials and Methods

Nematode populations, extraction, preservation and microscopic observations

Samples consisting of *A. arenaria* with sand attached to their root systems were collected from a Baltic coastal dune in Poland (54. 76623N; 17. 59239E). The nematodes were extracted by decantation and sieving and finally separated on extraction sieves. Adult specimens were fixed in a 4% TAF, transferred to pure glycerine and mounted on permanent microscope slides according to the method of Seinhorst (1959). Observations of morphological diagnostic features and morphometrical analysis were performed under a Leica light microscope using the Nomarsky differential interference contrast (DIC) technique. For molecular analyses, chosen females were subjected to microscopic analyses and then fixed in a mixture of dimethyl sulphoxide, disodium EDTA, and saturated NaCl (DESS) (Yoder *et al.* 2006). For comparison purposes, the study also included two Polish populations of *D. destructor* that were collected from Lubelskie (population 1) and Łódzkie (population 2) Voivodeships. Specimens were isolated from potato seeds using a modified Baermann technique (Brzeski *et al.* 1976). For morphological observations and molecular study, the investigated specimens were processed according to the methods described above.

Nematode lysis, amplification and sequencing

Ditylenchus destructor individual nematodes acquired from both the Baltic sand dune and potato seeds were washed in sterile water. The specimens were kept in water for two days and afterwards transferred to separate 0.2 ml polymerase chain reaction (PCR) tubes containing 25 µl of fresh, sterile water. Twenty-five µl of lysis buffer, as described by Holterman *et al.* (2006) was added and subsequently a lysis was performed at 65°C for 3 h, followed by a 5 min incubation at 100°C. The obtained single nematode lysates were used as DNA templates for PCR reactions or stored at –20°C. Lysis and all PCR reactions were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA).

Using the 988F/1912R and 1813F/2646R primer combinations (Holterman *et al.* 2006), 1,6 kbp long 18S rDNA was amplified in two overlapping fragments. Using the 61F primer combined with either 1006R, 1032R (approx. 900 bp) or MCB1R primer (700 bp), 28S rDNA was obtained (Holterman *et al.* 2006; Dobosz *et al.* 2013). The ITS rDNA was amplified with the F194/5368 primer combination (700 bp) (Cao *et al.* 2005). PCR reactions contained: 12.5 µl of Taq PCR Master Mix (2x) (EURx, Gdańsk, Poland) and 3,5 µl of Color Load dye (EURx, Gdańsk, Poland), 1 µl (5 µM) of forward and reverse primer each, 3 µl DNA template and H_2O to 25 µl total volume. The annealing temperature for all primer combinations was 54°C. Only in some cases in the amplification of the first part of the 18S rDNA fragment, 5 preliminary cycles at 45°C were applied (as in Holterman *et al.* 2006). After Midori Green (Nippon Genetics Europe, Duren, Germany) gel staining and gel electrophoresis, amplicons were visualized under UV illumination. Selected PCR products were cleaned from excess dNTPs and unincorporated primers using the Clean-Up Purification Kit (A&A Biotechnology, Gdynia, Poland).

Sequencing PCR reactions included: 2 µl BigDye Terminator v. 3.1 Ready Reaction Mix (ThermoFisher Scientific, Waltham, MA, USA), 1 µl BigDye 5X sequencing buffer (Thermo Fisher Scientific, Waltham, MA, USA), 1.6 µl (5 µM) forward primer and H_2O to 10 µl total volume. Sequencing reactions consisted of a denaturation step at 96°C for 1 min followed by 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 105 s. All rDNA fragments were subsequently sequenced with an ABI 3500xL genetic analyser (Applied Biosystems, Foster City, CA, USA) and deposited in GenBank under the following accession numbers: 18S rDNA: MN016947-8, 28S rDNA: MN016953-4, ITS rDNA: MN016967.

Phylogenetic analysis

The newly obtained ribosomal *D. destructor* sequences were analysed together with publicly available rDNA sequences (GenBank) using the BioEdit program v. 7.2.5 (Hall 1999). *Aphelenchoides fragariae* Christie, 1942 (Christie 1942) (in the case of the 18S rDNA data set) and *Bursaphelenchus xylophilus* Steiner and Bührer, 1934 (Steiner and Bührer 1934) (28S and ITS rDNA data sets) were used as outgroup species. The multiple alignments were generated by the ClustalW algorithm. The final multiple-sequence alignments comprised of 1703 overlapping characters in the case of 18S rDNA, 712 characters in the case of 28S rDNA and 622 in the case of ITS rDNA. The Bayesian phylogenies were constructed using the MrBayes v. 3.1 program (Ronquist and Huelsenbeck 2003). In the case of each data set, two independent runs were performed with four Markov chains per run. The program was run for 2,000,000 generations in each case and sample frequency was 100 generations. The sampled trees from each run were combined in a single 50% majority-rule tree. Stabilization of the likelihood and parameters was checked with the program Tracer v. 1.6 (Rambaut *et al.* 2014).

Results

Based on the analyses of morphological and morphometrical data, as well as the ribosomal sequences, specimens found in the root zone of *A. arenaria* were recognized as *D. destructor*. Morphometrical characteristics of this population and specimens of *D. destructor* isolated from potato seeds are given in Table 1. Females of *D. destructor* found in a dune population were characterized by the following features: an almost straight body, six incisures on the lateral field (Fig. 1A), low lip region, flattened and not offset from the rest of the body, a delicate stylet with small, posteriorly sloping knobs, pharyngeal basal bulb overlapping the intestine dorsally, ventrally or laterally at a length from 6.5 to 22.5 µm, a transverse vulva with raised lips, vulval lips with an annulated cuticle, length of post-uterine sac (2.9–4.0 times the body width at the vulva level and extending about 60% of the distance from vulva to anus) (Fig. 1B), a conical tail with a finely rounded terminus (Fig. 1C). Males are generally morphologically similar to females except for the reproductive system: spicules paired, arcuate with ventral tumulus, gubernaculum simple, bursa extending about 55% of the tail length.

Morphological and morphometrical characteristics of *D. destructor* specimens, representing populations developed in potato seed, agreed with those provided in literature. From the dune population the height of stylet knobs and the length of a hyaline part on the tail end differed (Table 1), as well as the arrangement of stylet knobs (laterally *vs* slightly posteriorly sloping) (Figs. 1D–E), the arrangement of the pharyngeal glands in relation to the intestine (dorsal or ventral *vs* dorsal, ventral or lateral) and the appearance of vulval lips (smooth *vs* annulated) (Figs. 1F–G).

From three independent nematode individuals of *D. destructor* from the sea dune 1,6 kbp long 18S rDNA identical sequences were obtained, while only one 18S rDNA fragment (only first fragment – approx. 900 bp) was acquired from *D. destructor* from potato seeds. The sequence differed (by having four nucleotides along the obtained fragment) from the sequences acquired from the coastal population. In the case of the 28S rDNA, three sequences from independent nematode individuals were acquired for each *D. destructor* population (sand dune and potato). There were four single nematode polymorphisms (SNPs) found between 28S rDNA sequences acquired from the sand dune and the potato seed populations. ITS sequences were obtained only for *D. destructor* from the dune.

All three phylogenetic analyses (Figs. 2–4) showed that *D. destructor* from the Baltic dune belonged to the *D. triformis* group, particularly the *D. destructor* lineage, although it was slightly divergent from the two distinguished *D. destructor* subclades.

Discussion

The morphology and morphometry of *D. destructor* specimens found on the potato and among the roots of *A. arenaria* were broadly consistent with previous descriptions in the literature (Thorne 1945; Goodey 1952; Wu 1958; Fujimura *et al.* 1986; Koliopanos and Vovlas 1977; Brzeski 1991, 1998; Zhang and Zhang 2007; Yu and Zaida 2012; Mwaura Mutua 2014; Ou *et al.*

Table 1. Morphometrical characteristic of *Ditylenchus destructor*. Measurements in micrometers (µm) in the form mean ± standard deviation (range)

Population	*D. destructor*-1	*D. destructor*-2	*D. destructor*-3
	Females		
n	37	15	30
Locality	Poland	Poland	Poland
Plant	*A. arenaria*	potato	potato
Character/ratio			
*L**	1002 ± 107 (882–1219)	1163 ± 94 (1013–1304)	1064 ± 104 (869–1207)
a	43.6 ± 4.5 (35.2–50.1)	40.2 ± 2.9 (35.2–44.3)	34.9 ± 3.7 (30–45.3)
b	6.8 ± 0.9 (5.8–8.1)	7.6 ± 0.9 (5.9–9.2)	7.3 ± 1 (6.1–9.3)
b′	6.1 ± 0.7 (5.4–8.3)	6.8 ± 0.9 (5.3–8.7)	6.6 ± 1 (4.7–8.5)
*c**	14.2 ± 1.5 (11.6–17.1)	16.2 ± 0.8 (14.1–18.2)	14.1 ± 1.4 (11.9–17)
c′	4.7 ± 0.5 (3.7–5.3)	3.8 ± 0.5 (3.2–4.7)	4.1 ± 0.5 (3.3–5.3)
V	80.3 ± 2.1 (70.7–82.9)	81.6 ± 3.3 (79.3–83.3)	84.9 ± 3.3 (76.7–88.4)
Stylet length	12.2 ± 0.4 (11.3–12.7)	10.8 ± 0.5 (10.1–11.6)	11.2 ± 0.4 (10.2–11.9)
Length of cone/total stylet length*	45.8 ± 3.2 (39.3–50.4)	47.4 ± 3.5 (41.7–56.8)	46.4 ± 2.6 (43.5–50)
High of stylet knobs	2.4 ± 0.2 (2.1–2.9)	1.6 ± 0.09 (1.4–1.7)	1.6 ± 0.2 (1.3–1.8)
Number of lateral lines*	6	6	6
Posterior bulb*	13.5 ± 4.2 (5–21.7)	18.5 ± 5.1 (7.8–26)	14.3 ± 3.9 (8–20)
Postvulval uterine sac (PUS) length	73.5 ± 11 (61–91.5)	95.5 ± 12.4 (71–111)	97.5 ± 14 (75–117)
PUS/Vulva-anus%*	60.9 ± 6.8 (49.2–75.2)	67.1 ± 10 (53–89)	72.3 ± 3.9 (59–84)
Vulva-anus length* [%]	173 ± 15 (146–207)	198 ± 19 (155–239)	184 ± 22 (138–234)
Length of hyaline	1.2 ± 0.2 (0.8–1.8)	2 ± 0.6 (1–2.9)	2 ± 0.6 (1–2.9)
	Males		
n	25	10	30
L	1000 ± 157 (808–1413)	1031 ± 84 (955–1186)	1018 ± 78 (892–1161)
a	46.5 ± 6.1 (37.1–58.6)	42.2 ± 6.2 (33.5–50.2)	42.5 ± 4.2 (35.5–54.0)
b	6.8 ± 1.2 (5.0–10.8)	7.2 ± 0.8 (5.6–8.3)	7.5 ± 0.7 (6.9–9.2)
b′	6.2 ± 0.9 (4.7–8.6)	6.6 ± 0.5 (5.9–7.3)	6.9 ± 0.2 (6.3–8.3)
c	13.9 ± 1.2 (12.6–16.1)	15.3 ± 0.5 (14.9–16.5)	14.0 ± 1.3 (12.5–17)
c′	4.5 ± 0.4 (3.8–5.1)	3.9 ± 0.3 (3.5–4.4)	4.4 ± 0.5 (3.9–5.2)
Stylet length	11.9 ± 0.4 (11.1–12.4)	10.5 ± 0.3 (10.0–11.0)	11.1 ± 0.4 (10.1–11.7)
High of stylet knobs	2.3 ± 0.2 (2.0–2.6)	1.5 ± 0.08 (1.4–1.6)	1.66 ± 0.15 (1.4–1.8)
Length of hyaline	1.1 ± 0.2 (0.8–1.6)	2.2 ± 0.5 (1.5–2.9)	1.6 ± 0.3 (1.2–2.3)
Spikule length*	26.0 ± 1.1 (24.6–27.4)	26.1 ± 2.1 (23.2–29.1)	25.5 ± 1.2 (24.0–27.9)

D. destructor-1 – population of *D. destructor* isolated from root system of *Ammophila arenaria* (L.); *D. destructor*-2 and *D. destructor*-3 – populations isolated from potato seeds; *n* – number of specimens; *L* – body length; *a* – ratio of body length to largest body with; *b* – ratio of body length to pharynx length from head to pharyngeo-intestinal junction; *b′* – ratio of body length to pharynx length from anterior end to posterior end of glandular lobe; *c* – ratio of body length to tail length; *c′* – ratio of tail length to body with at anus level; *V* – distance from anterior end to vulva expressed in percent of body length

*the features indicated by EPPO (2017)

2017). Analysis of the studied material allowed us to broaden the knowledge about the variability range of some morphological features of this economically important species.

The shape and arrangement of stylet knobs of *D. destructor* from the dune were also observed in other potato rot nematode populations (Thorne 1961; Paramonov 1962, 1970; Brzeski 1998; Zheng *et al.* 2016). The height of stylet knobs of this species was defined for the first time in this publication. Analysis of this feature indicated that it was stable in the population of both females and males (cv < 10%).

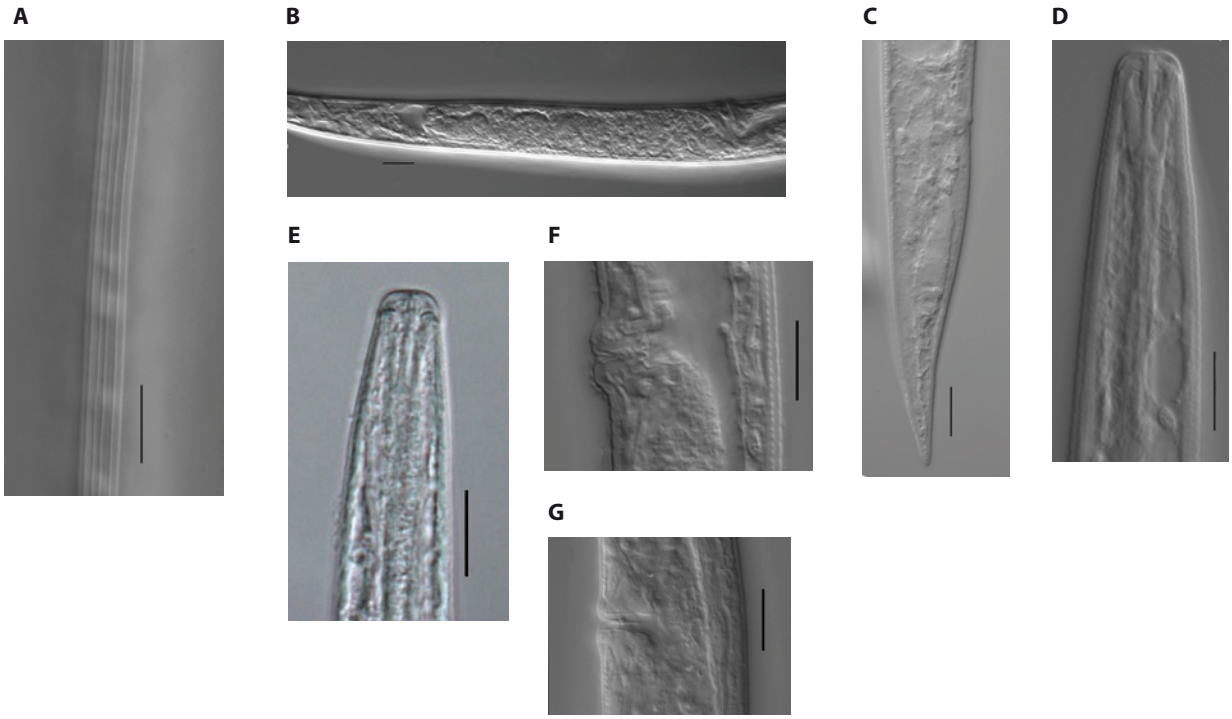

Fig. 1. *Ditylenchus destructor* (A) lateral field of female from *Ammophila arenaria*, (B) postvulval uterine sac of female from *A. arenaria*, (C) tail of female from *A. arenaria*, (D) stylet of female from *A. arenaria*, (E) stylet of female isolated from potato, (F) vulval lips of female from *A. arenaria*, (G) vulval lips of female from potato. Scale = 10 µm

The type of pharyngeal gland position, in relation to the intestine, has been used to date as a distinguishing feature for economically important species in the genus *Ditylenchus* (EPPO 2017). In the population of *D. destructor* found on the dune, a high variability in the positioning of these glands was observed. In addition to specimens in which the pharyngeal glands overlap the intestine on the dorsal side, there were also those with glands overlapping the intestine on the ventral or lateral side. The variability of this feature was earlier explained as a result of the host plant acting on the nematode (Thorne 1961). However, the length of the glandular part overlapping the intestine of the *D. destructor* from the dune (except for one female) did not exceed the value of one body width at the level of the pharyngo-intestinal valve. It is consistent with Hooper's (1973) data.

In this article, for the first time, it was noted that the vulval lip cuticle in *D. destructor* can be smooth or have a slightly transverse striation. Earlier observations of this species were concentrated mainly on the gonad construction (Goodey 1952; Wu 1958).

Comparing the population from the dune and the potatoes, for the first time attention was paid to the hyaline part of the tail length. Despite the slightly higher mean values observed in the populations isolated from the potato, this feature was not constant for the population. The coefficient of variation reached values between 17.2 and 32.5%.

The performed phylogenetic studies based on three ribosomal regions (18S, 28S and ITS rDNA) confirmed the identity of the analyzed species found in the Baltic sand dune as *D. destructor*. In each case, the obtained rDNA fragments were positioned in the well-supported clade that included other representatives of this species (Figs. 2–4).

Due to the fact that *D. destructor* is a pest of economically important plants, the populations described so far came from arable land soils. Reports on the species occurrence in the natural environment (Smart 1959) came from the Arkansas (USA) area, where it was isolated from the soil around the grass roots *Stenotaphrum secundatum* (Walter) Kuntze and from the research described in this article.

The present results show that *D. destructor* can develop in such a specific natural environment as the seaside dunes. In spite of the large numbers of potato rot nematodes, grass root damage has not been observed, it can be concluded that these plants could serve as a natural reservoir of the *D. destructor* population. Smart's research (1959) also showed that when the specimens of this species were transferred to potato tubers, they proved to be pathogenic for the plants.

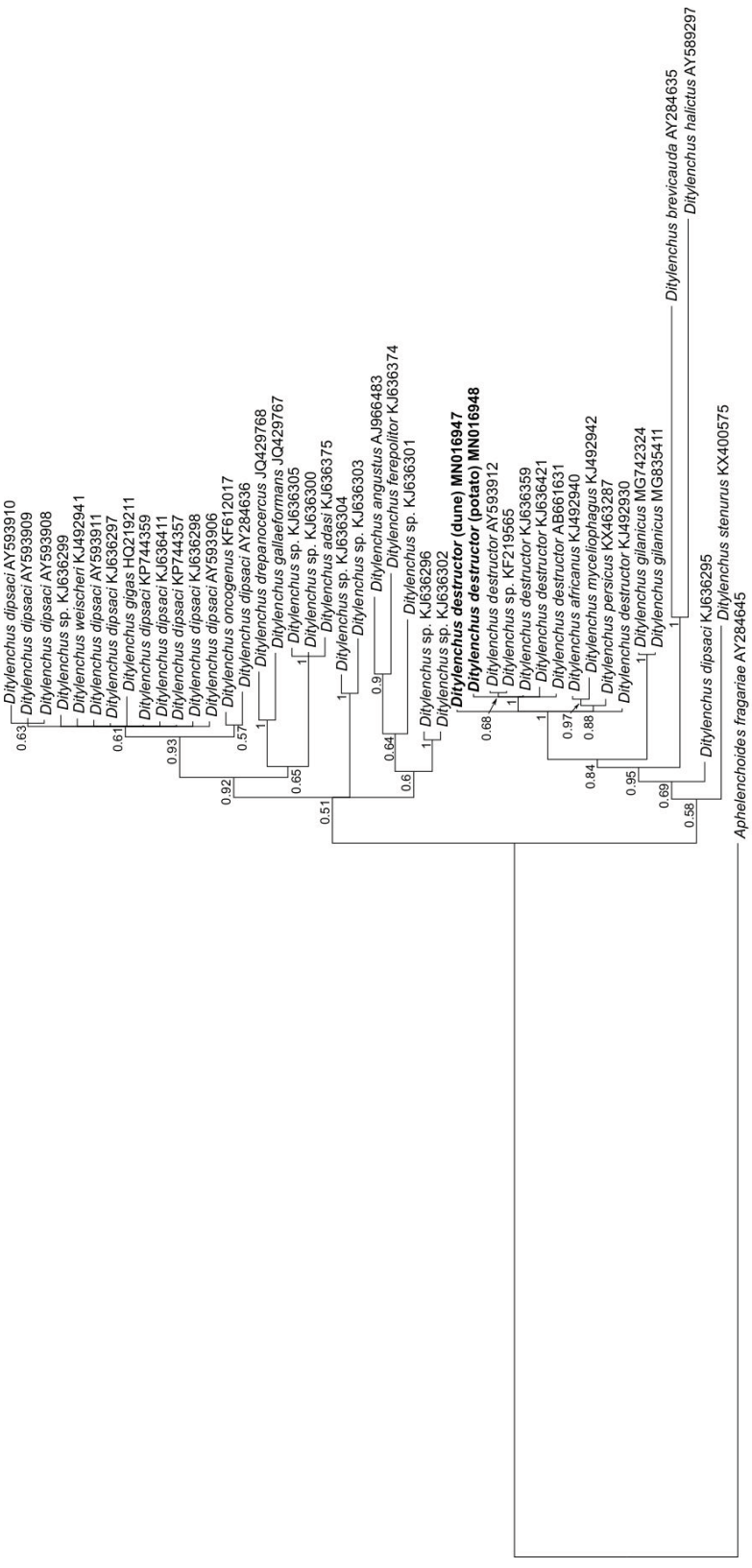

Fig. 2. Bayesian phylogenetic tree of the genus *Ditylenchus* inferred from 18S rDNA sequences. Newly acquired sequences are in boldface font

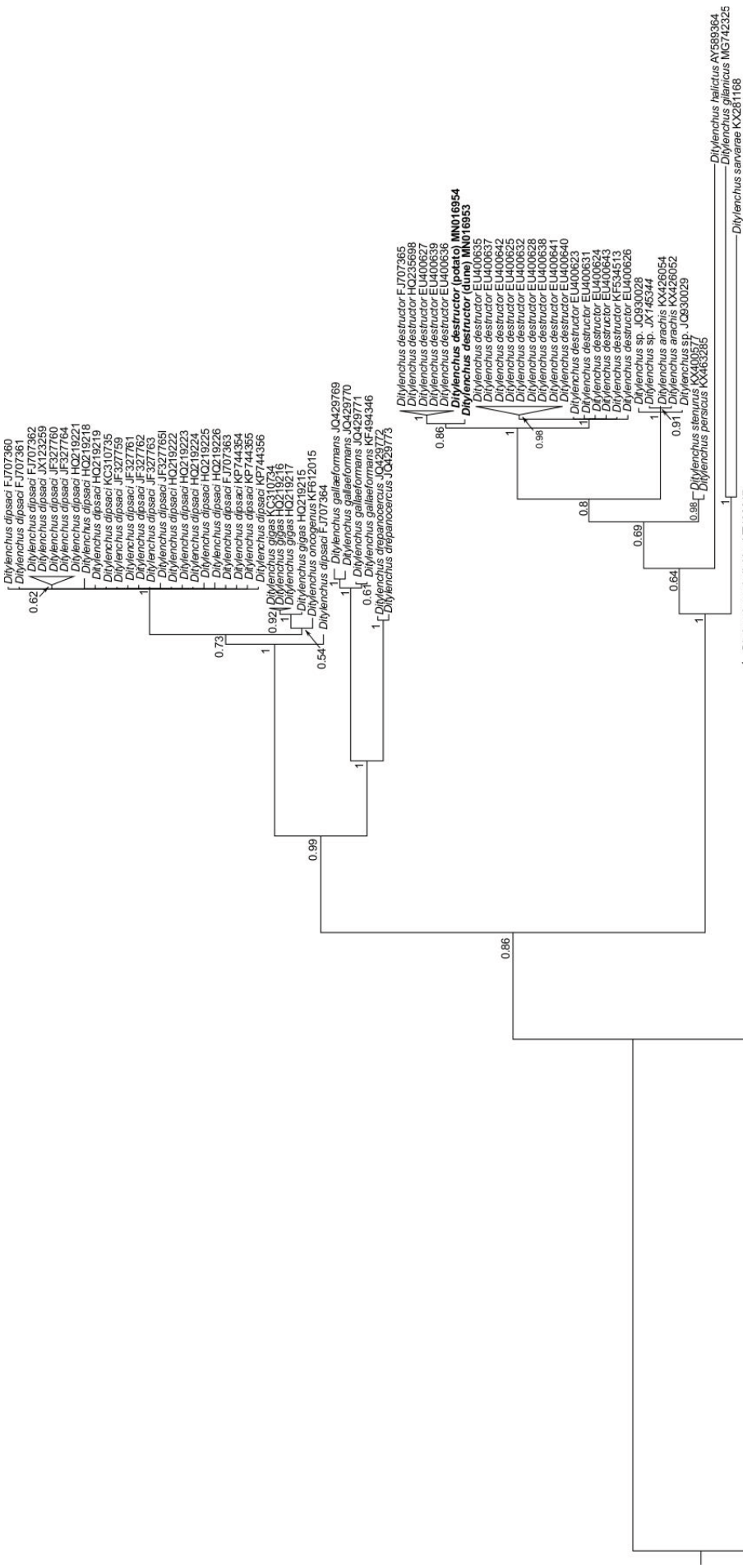

Fig. 3. Bayesian phylogenetic tree of the genus *Ditylenchus* inferred from 28S rDNA sequences. Newly acquired sequences are in boldface font

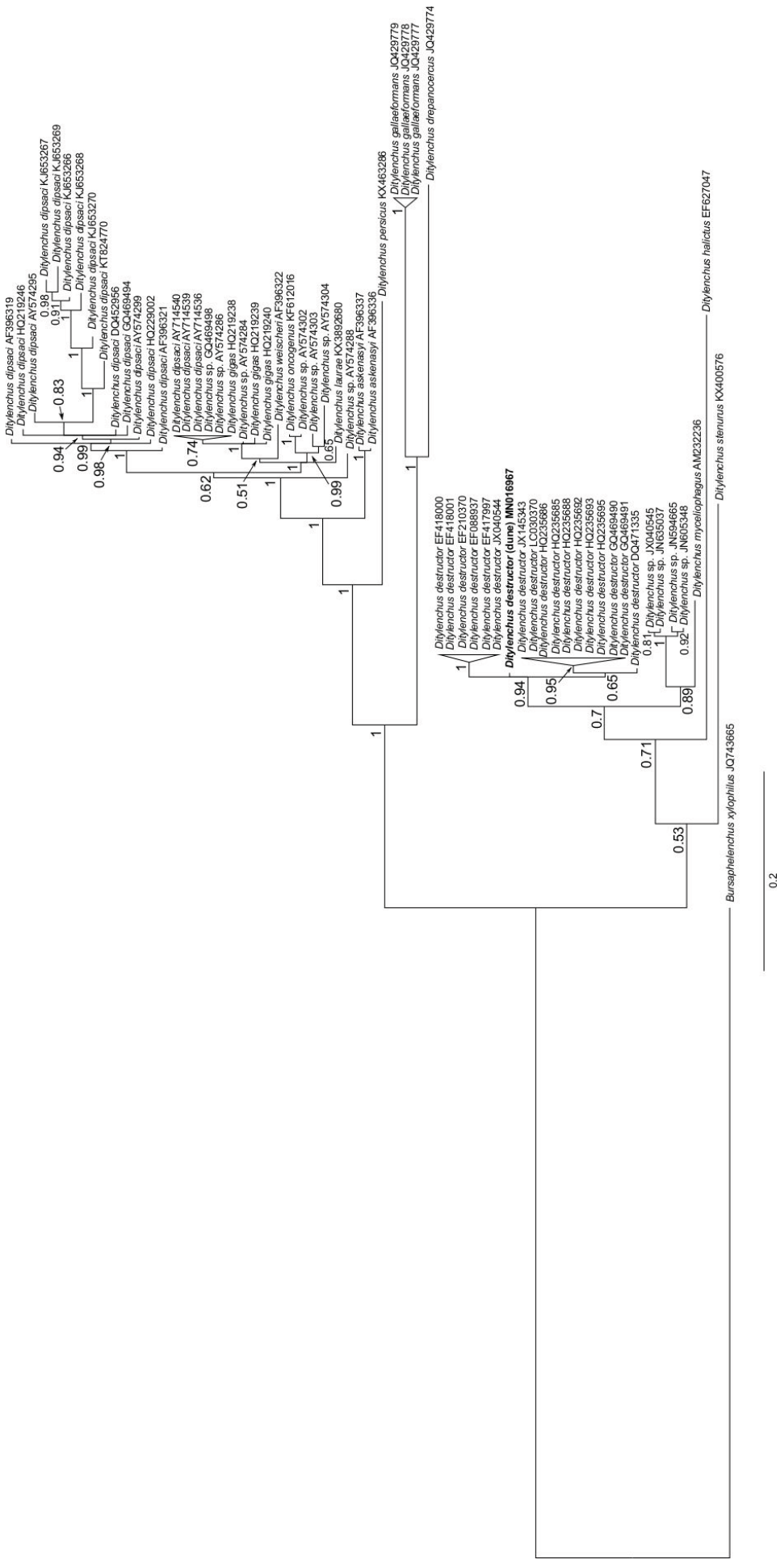

Fig. 4. Bayesian phylogenetic tree of the genus *Ditylenchus* inferred from ITS rDNA sequences. Newly acquired sequences are in boldface font

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