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Nematology 19 (2017) 197-209

Nematology
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Ditylenchus laurae sp. n. (Tylenchida: Anguinidae) from Poland – a new species of the *D. dipsaci* complex associated with a water plant, *Potamogeton perfoliatus* L.

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Received: 14 September 2016; revised: 28 November 2016
Accepted for publication: 28 November 2016; available online: 10 January 2017

Summary – The genus *Ditylenchus* consists of more than 60 species, some of which are plant parasites. In this paper we report on *Ditylenchus laurae* sp. n. from Poland, a new species associated with the aquatic plant, *Potamogeton perfoliatus* L. *Ditylenchus laurae* sp. n. is characterised by a long and slender body with L = 1881 (1523-2095) μm and 1875 (1690-2089) μm, a = 88.8 (72.5-102.5) and 89.7 (71.9-97.9), stylet length = 10.8 (9.6-12.1) μm and 8.3 (7.7-9.0) μm, tail length = 117.2 (103.5-126.7) μm and 102.4 (98.3-113.6) μm in females and males, respectively; four incisures, rounded stylet knobs, long basal bulb (about ten times as long as wide), post-vulval uterine sac from 4.3-5.6 vulval body diam. long, and mucronate tail. Characterisation using the ITS rRNA, *COI* and *hsp*90 gene sequences was conducted and a phylogenetic analyses revealed that *D. laurae* sp. n. belongs to the *D. dipsaci* complex.

Keywords – aquatic plant, *COI*, *hsp90*, ITS rRNA, molecular, morphology, morphometrics, new species, phylogeny, Potamogetonaceae, taxonomy.

The genus *Ditylenchus* Filipjev, 1936 consists of more than 60 species, most of which are mycetophagous although several are plant parasites (Brzeski, 1991; Siddiqi, 2000; Vovlas *et al*., 2011). However, despite the limited number of plant-parasitic representatives in the genus, they do include important agricultural pests, such as *D. africanus* Wendt & Webster, 1995, *D. angustus* (Butler, 1913) Filipjev, 1936, *D. arachis* Zhang, Liu, Janssen, Zhang, Xiao, Li, Couvreur & Bert, 2014, *D. destructor* Thorne, 1945, *D. dipsaci* (Kühn, 1857) Filipjev, 1936 and *D. gigas* Vovlas, Troccoli, Palomares-Rius, De Luca, Liébanas, Landa, Subbotin & Castillo, 2011. The type species of the genus is a stem and bulb nematode, *D. dipsaci*, which is distributed worldwide and is a pest of many crops and also parasitises weeds. Phylogenetic analysis of ITS rRNA gene sequences of different populations and races of stem and bulb nematode (Subbotin *et al*., 2005) revealed several cryptic species previously identified under the name '*D. dipsaci*'. Presently, the *D. dipsaci* species complex contains several morphologically similar and phylogenetically related species: *D. dipsaci sensu stricto*, *D. weischeri* Chizhov, Borisov &

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DOI 10.1163/15685411-00003040

Subbotin, 2010, *D. gigas*, *D. oncogenus* Vovlas, Troccoli, Palomares-Rius, Cantalapiedra-Navarrete, Liébanas, Landa, Subbotin & Castillo, 2016, and three as yet undescribed species, namely *Ditylenchus* sp. D from *Pilosella* spp., *Ditylenchus* sp. E from *Crepis praemorsa* (L.) Tausch, and *Ditylenchus* sp. G from *Plantago maritima* L. All of these plant-parasitic species were found associated with land plants and only *D. tobaensis* (Schneider, 1937) Kirjanova, 1951, was associated with aquatic vascular plants (Schneider, 1937). Schneider (1937) described *D. tobaensis* (= *Tylenchus dipsaci var. tobaensis*) inducing galls in *Myriophyllym spicatum* L. and *Potamogeton malaianus* Mig. growing submersed in the Toba Lake, North Sumatra. Zeller (1937) gave descriptions of the galls on both plant species and found that nematode-infected *P. malaianus* exhibited shortened internodes, increased branching and reduced leaf development (Gerber & Smart, 1987). However, this species was not accepted as valid by most authors and is presently considered either as a synonym of *D. dipsaci* (Brzeski, 1991; Siddiqi, 2000), or a *species inquirendum* (Sturhan & Brzeski, 1991; Siddiqi, 2000).

In this paper we report a new species, *D. laurae* sp. n., found associated with a fully submerged water plant, the clasping leaf pondweed, *Potamogeton perfoliatus* L. The present study provides morphological and molecular characterisation of this species and analyses of its relationships with other species of the *D. dipsaci* complex.

Materials and methods

Extraction, fixation and mounting of specimens

Specimens of the new species were extracted from plants collected on 12 December 2012. Plant rhizomes were cut into pieces about 2 cm long and left on sieves (100 μm mesh) submerged in water in Petri dishes. After 24-48 h, the nematodes that had emerged were used for study. Several specimens were preserved in DESS (Yoder *et al*., 2006) for the molecular study. The remaining specimens were heat-killed, preserved in TAF (Courtney *et al*., 1955) and transferred to glycerin as described by Seinhorst (1959). Specimens were studied and documented using a Leica DM 5000 compound microscope.

Scanning electron microscope study

For scanning electron microscope (SEM) observations the nematodes were fixed overnight at 5°C in a solution of 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. The samples were then washed three times with a 0.05 M buffer solution for 15 min. Post-fixation was carried out in 1% osmium tetroxide in the same buffer for 2 h. After fixation the samples were rinsed in distilled water three times (5 min) and dehydrated in an ethanol series (10, 30, 50, 70, 80, 90, 96, 100%). Dehydrated specimens were critical point-dried in CO_2 (Leica EM CPD300) and transferred with an eyebrow hair mounted on a wooden probe onto an SEM stub covered with a double-sided adhesive carbon disc. The samples were coated with gold/palladium and viewed under high vacuum in a Hitachi S3000N SEM equipped with a secondary electron detector.

DNA isolation, PCR amplification and sequencing

DNA was isolated from nematodes as described by Jeszke *et al*. (2015). Purified DNA was used as template for PCR with primer pairs amplifying ITS rRNA, partial *COI* mtDNA and partial *hsp90* gene fragments. The S18 (5′-TTG ATT AGG TCC CTG CCC TTT-3′) and S26 (5′-TTT CAC TCG CCG TTA CTA AGG-3′) primer set was used for amplification of 18S (partial)-ITS1-5.8S-ITS2-(partial) 28S rRNA gene fragment. PCR was done as described previously (Marek *et al*., 2010; Jeszke *et al*., 2015). The new ditCOIFw (5′-CAC CYG AYA TGT CCC TC-3′) and ditCOIRw (5′-GTG TAC ATR TGG TGR GC-3′) primer set was designed to amplify a partial *COI* mtDNA gene. PCR was performed in 10 μl volume containing 1 μl DNA template, 1 μM of each primer, 5 μl of DreamTaq Master Mix (Thermo Scientific) and sterile distilled water. The amplification conditions were as follows: denaturation for 3 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 58°C, 40 s at 72°C; and final extension for 5 min at 72°C. The new Hsp90_ditF (5′-GCG CAA TCC GGM YGA NAC SAA TGA-3′) and Hsp90_ditR (5′-GAA WGG AGC ACG YTG AGG CAC GAA-3′) primer set was also designed to amplify a partial *hsp90* gene. PCR was performed as described by Tanha Maafi *et al*. (2003).

PCR products were separated on 1% agarose gel and PCR products were excised from the gel followed by elution using QIAquick® Gel Extraction Kit (Qiagen). Subsequently, products were cloned and transformed

to *E. coli* cells as described previously (Jeszke *et al*., 2014). Recombinant plasmids were purified from bacterial colonies using QiaPrep Spin Miniprep Kit (Qiagen) and sequenced. The new sequences of *D. laurae* sp. n. were deposited in the GenBank database under accession numbers: KX421380-KX421382 for partial *COI* gene, KX389268 for ITS rRNA gene and KX756631-KX756633 for partial *hsp90* gene. Additionally, several sequences of the partial *COI* gene from *D. dipsaci* and *D. gigas* were also included in this study. The *D. dipsaci* sequences were obtained from six Polish populations associated with *Allium cepa* L. (deposited in GenBank under accession numbers: KT072576 and KT072579-KT0725781) and *Phlox paniculata* L. (GenBank: KT072577, KT072578). Two sequences of a *D. gigas* population from *Vicia faba* L. were also obtained, one from a Polish population (KT072582) and one from a population collected in the UK (KT072583). Except for the UK population, all these *COI* gene sequences were obtained from DNA isolates used in the study of Jeszke *et al*. (2014). Populations of *D. dipsaci* and *D. gigas* were identified on the basis of morphology and confirmed by molecular analysis using the ITS rRNA gene sequences.

Phylogenetic and sequence analysis

The newly obtained sequences of the ITS rRNA, *COI* mtDNA and *hsp*90 genes were aligned using ClustalX 1.83 with default parameters with their corresponding published gene sequences of species from the *D. dipsaci* complex and outgroups (Subbotin *et al*., 2004, 2005; Marek *et al*., 2005, 2010; Kerkoud *et al*., 2007; Chizhov *et al*., 2010; Vovlas *et al*., 2011, 2016; Jeszke *et al*., 2014, and others). Sequence alignment was manually edited using GenDoc 2.5.0. Pairwise divergence between taxa was calculated as the absolute distance value and the percent of mean distance, with adjustment for missing data, using PAUP* 4b10 (Swofford, 2003). Sequence datasets were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The best fit model of DNA evolution was obtained using the program JModeltest v. 0.1.1 (Posada, 2008) with the Akaike Information Criterion. BI analysis for each gene was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. 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 on appropriate clades. The ITS and *COI* sequence alignments were also used to construct phylogenetic minimum spanning networks using PopARTsofware (http://popart.otago.ac.nz) (Bandelt *et al*., 1999).

Results

Ditylenchus laurae[*] sp. n.
(Figs 1-3)

Measurements

See Table 1.

Description

Female

Body from almost straight to moderately arcuate (Fig. 1A). Cuticle fine annulated (Fig. 2B), annuli 0.8-1.1 μm wide. Lateral field with four lines. Under LM, distance between inner lines larger than between external and internal incisures (Fig. 1H); under SEM, however, lines almost equidistant (Fig. 2B-D). Under LM, lines almost invisible, observed in only five specimens. In SEM, lines close to vulval level irregular (Fig. 2E). Lip region anteriorly flattened, not offset from rest of body (Fig. 3A, B). Lips annuli indistinct under LM, visible under SEM (Fig. 2A). Six lips visible under SEM (Fig. 2A), indistinct under LM. Stoma opening pore-like, situated in middle of a slightly raised oral disc. Stylet delicate, conus shorter than shaft. Stylet knobs rounded in most specimens, rarely slightly sloping posteriad (Fig. 3B). Dorsal gland orifice close to knobs (Fig. 3B). Metacorpus (median bulb) oval, 2.5-3.0 times as long as wide. Valve situated centrally to slightly anteriorly. Isthmus narrow and long, *ca* 2.0-2.3 times longer than procorpus. Nerve ring surrounding isthmus in anterior half. Basal bulb very elongated, *ca* ten times longer than wide (Fig. 3A, D). No overlap with intestine or a small overlap (up to half of corresponding body diam.) in a few specimens. Reproductive system prodelphic, ovary outstretched with oocytes in a single row. Spermatheca elongated, filled with sperm. Vulva a transverse slit, lips more or less protruding above body contour, without annulation. Vagina straight, perpendicular to body axis, occupying less than half of corresponding body diam. (Fig. 3G). Post-vulval uterine sac (PUS) well

[*] The species was named in honour of Laura Skwiercz, a grand-daughter of first author (AS).

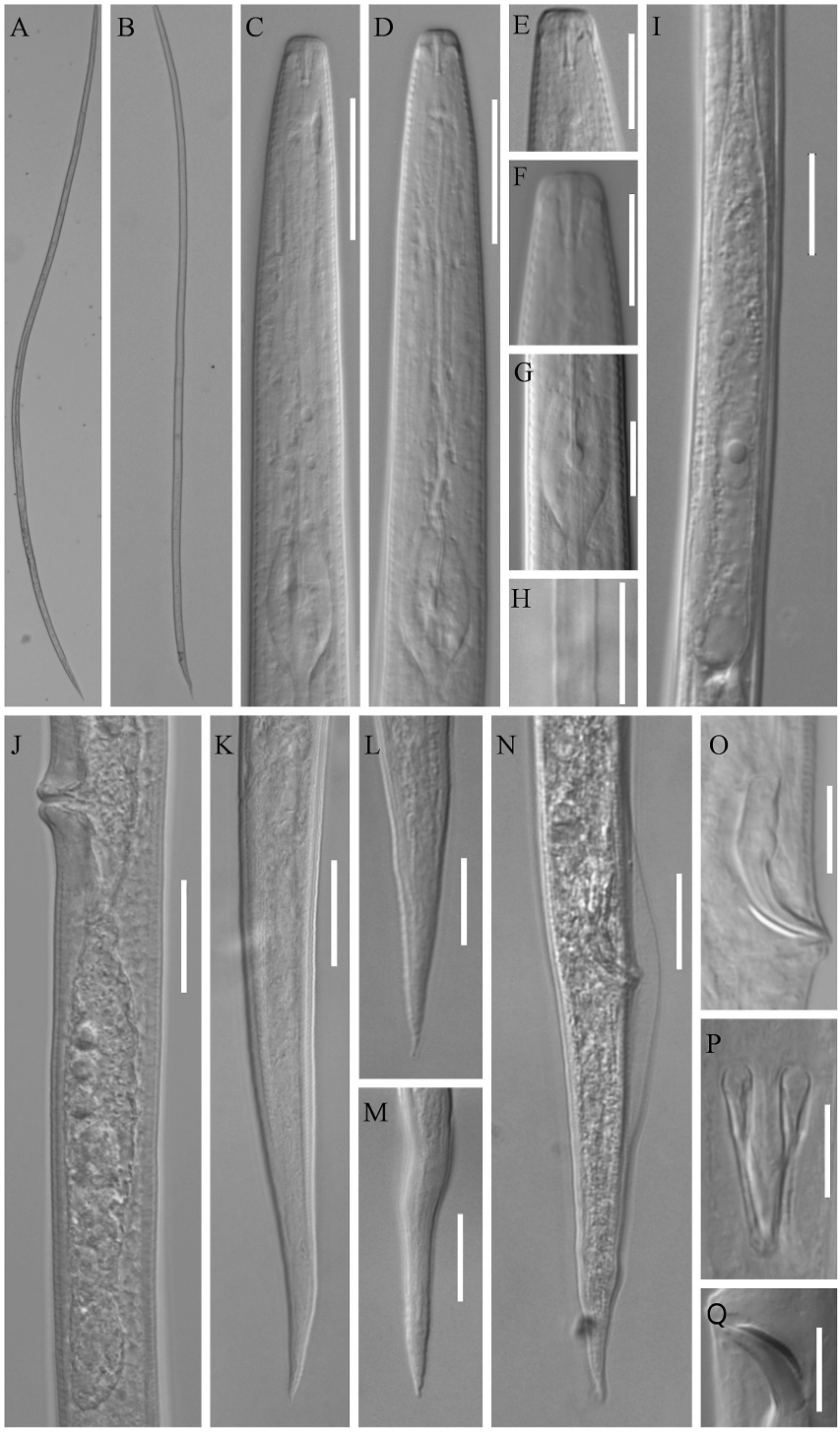

Fig. 1. Light microscope photographs of *Ditylenchus laurae* sp. n. A, B: Body posture of female and male, respectively; C, D: Anterior body part of female and male, respectively; E, F: Stylet and lip region; G: Metacorpus; H: Lateral field with four lines; I: Basal bulb; J: Vulva and post-vulval uterine sac; K: Female tail; L, M: Variation in tail tip; N: Male tail with focus on bursa; O, P: Spicules in lateral and dorsal view, respectively; Q: Gubernaculum. (Scale bars: A-D, I-K, N = 20 μm; E-H, L, M, O-Q = 10 μm.)

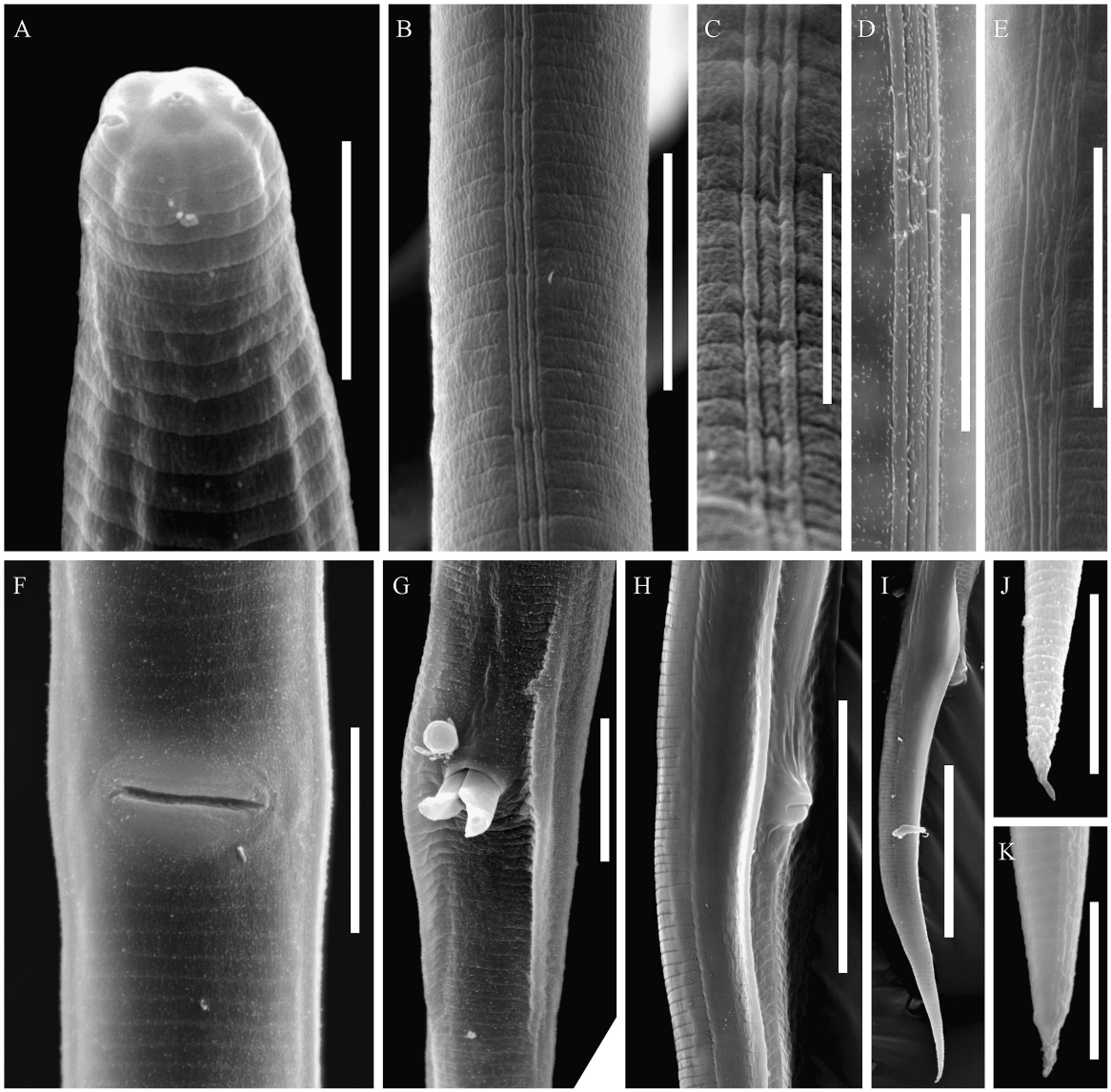

Fig. 2. SEM photographs of *Ditylenchus laurae* sp. n. A: Lips and anterior body region; B: Cuticle annulation and lateral field at about 25% of body length from anterior end; C, D: Lateral fields; E: Distorted lateral field at vulval level; F: Vulva in ventral view; G: Spicules, cloaca and bursa in dorsal view; H: Cloacal region and bursa in lateral view; I: Male tail and bursa in lateral view; J, K: Tail tips with mucron. (Scale bars: A, C = 5 μm; B, D-G, H = 30 μm; I = 10 μm; J, K = 20 μm.)

developed, elongated, 4.3-5.6 vulval body diam. long, occupying 38.5 (31.9-45.7)% of vulva-anus distance (Fig. 3F). Tail conoid, elongated, tapering posteriorly to terminus bearing a more or less pronounced mucron (Fig. 3H, I).

Male

Less frequent than female, sex ratio *ca* 1:5. Morphology of anterior body part similar to female (Figs 1B, D; 3C). Spicules paired, arcuate (Fig. 1OP). Gubernaculum simple, *ca* one-third of spicule length long (Fig. 1Q). Bursa leptoderan extending anteriorly to cloacal aperture for *ca* 1.5 cloacal body diam., covering half or slightly less of tail length (Figs 1N; 2I). Tail elongate-conoid, straight, tapering gradually to terminus bearing a more or less pronounced mucron (Figs 1N; 3J).

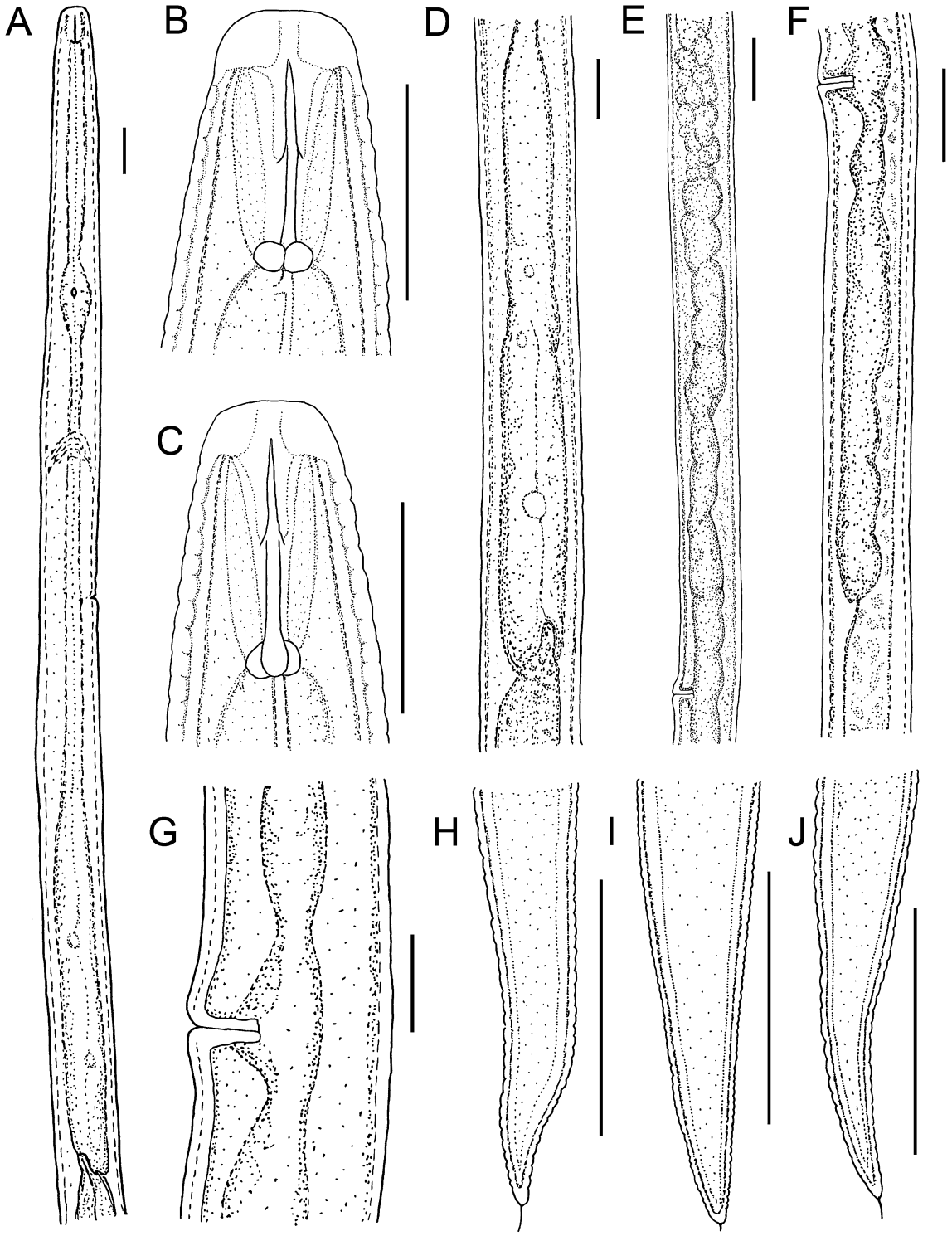

Fig. 3. Line drawings of *Ditylenchus laurae* sp. n. A: Anterior body region; B, C: Lips and stylet of female and male, respectively; D: Basal bulb of pharynx; E: Anterior genital tract; F: Post-vulval uterine sac; G: Vulva; H, I: Female tail tip with mucron; J: Male tail tip with mucron. (Scale bars = 10 μm.)

Table 1. Morphometrics of *Ditylenchus laurae* sp. n. from Poland. All measurements are in μm and in form mean ± s.d. (range).

Character	Female		Male
	Holotype	Paratypes	Paratypes
n	1	17	5
L	2078	1881 ± 137.3 (1523-2095)	1875 ± 176.7 (1690-2089)
a	91.1	88.8 ± 7.4 (72.5-102.5)	89.7 ± 10.7 (71.9-97.9)
b	6.6	6.2 ± 0.7 (5.5-8.3)	5.9 ± 1.0 (4.3-6.7)
c	17.6	16.1 ± 1.1 (14.4-18.2)	18.3 ± 1.7 (16.9-21.2)
c′	7.7	7.7 ± 0.7 (6.7-8.9)	6.2 ± 0.5 (5.7-6.9)
V	78.7	79.4 ± 1.1 (76.9-81.7)	–
Lip diam.	6.6	6.2 ± 0.3 (5.6-6.6)	6.1 ± 0.3 (5.8-6.5)
Lip height	2.2	2.3 ± 0.2 (2.0-2.7)	2.2 ± 0.4 (1.9-2.6)
Stylet length	9.3	10.8 ± 0.7 (9.6-12.1)	8.3 ± 0.6 (7.7-9.0)
Stylet conus length	4.1	5.1 ± 0.5 (4.1-5.9)	5.4 ± 0.9 (4.2-6.2)
Stylet shaft length	5.3	5.7 ± 0.5 (4.7-6.8)	6.0 ± 0.5 (5.5-6.7)
Stylet knobs width	2.8	2.3 ± 0.3 (2.0-2.8)	2.3 ± 0.07 (2.2-2.4)
Metacorpus width	9.9	9.4 ± 0.4 (8.9-10.0)	9.5 ± 0.5 (9.0-10.3)
Anterior end to metacorpus centre	86	80 ± 2.5 (76-86)	88 ± 10.2 (80-104)
Anterior end to excretory pore	181	174 ± 7.0 (158-182)	163 ± 11.7 (148-178)
Pharynx (to cardia)	316	304 ± 23.5 (253-347)	323 ± 44.1 (287-399)
Vulva to anus distance	324	270 ± 35.3 (200-308)	–
Diam. at vulval level or mid-body	22.8	21.4 ± 2.8 (17.7-28.9)	21.0 ± 1.8 (19.3-23.8)
Post-uterine sac	121	103 ± 14.25 (80-136)	–
Spicules	–	–	26.6 ± 2.5 (23.4-28.8)
Gubernaculum	–	–	9.2 ± 1.5 (8.1-10.9)
Tail length	118	117 ± 6.8 (103-127)	102 ± 6.4 (98-114)
Anal body diam.	15.4	15.3 ± 1.2 (13.1-17.5)	16.6 ± 0.8 (16.0-17.8)

NOTE

Karssen & Willemsen (2010) concluded that the presence of a ventral tumulus in the calomus area of the *D. destructor* spicule is a very useful character to distinguish it from *D. dipsaci*, even if the spicule is not in a perfectly lateral position. According to our observation (Fig. 1O) the spicules of our new species are similar to those of *D. destructor*.

TYPE HOST AND LOCALITY

Collected by the first author (AS) on 12 December 2012 from a clasping leaf pondweed, *Potamogeton perfoliatus* L. (Potamogetonaceae), located close to Rewa village, Pomeranian Voivodeship, Poland.

TYPE MATERIAL

Holotype female, three paratype females and three paratype males deposited in the Museum and Institute of Zoology, Polish Academy of Sciences, Warsaw, Poland. Accession numbers: holotype MIZ 1/2016/1; paratype females MIZ 1/2016/5-1/2016/7; paratype males MIZ 1/2016/2-MIZ 1/2016/4. Seven paratype females and one paratype male deposited in the Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain. Paratype female accession numbers: NIAS_201601-NIAS_201607; paratype male: NIAS_201608. Remaining seven paratype females and one paratype male deposited in the Wageningen Nematode Collection, Wageningen, The Netherlands. Paratype female accession numbers: WT 3676-WT 3682; paratype male: WT 3683.

DIAGNOSIS AND RELATIONSHIPS

Ditylenchus laurae sp. n. is characterised by a long and slender body with L = 1881 (1523-2095) μm and 1875 (1690-2089) μm, a = 88.8 (72.5-102.5) and 89.7 (71.9-97.9), stylet length = 10.8 (9.6-12.1) μm and 8.3 (7.7-9.0) μm, tail length = 117 (103-127) μm and 102.4 (98.3-113.6) μm in female and male, respectively; presence of four incisures, rounded stylet knobs, very long basal

bulb (*ca* ten times as long as wide), PUS from 4.3-5.6 vulval body diam. long, and tail with small mucron. This combination of traits, particularly the long body, long tail and high ratio a, enable the rapid and unambiguous diagnosis of *D. laurae* sp. n. from the majority of both myceliophagous and plant-parasitic species of the genus.

Species of greater morphological and morphometric similarity include *D. gigas*, *D. dipsaci*, *D. weischeri* and *D. tobaensis*. From these species *D. laurae* sp. n. can be differentiated as follows: from *D. gigas* by more slender body (a = 88.8 (72.5-102.5) *vs* 48.9 (43-56.4) and 89.7 (71.9-97.9) *vs* 56.7 (34.3-63) in female and male, respectively), stylet knobs rounded or slightly sloping posteriad *vs* distinctly sloping posteriad, bursa covering tail for half or slightly less of its length *vs* 72-75%, and tail tip with short mucron *vs* finely rounded (Vovlas *et al*., 2011); from *D. dipsaci* by more slender female body (a = 88.8 (72.5-102.5) *vs* 30-64), female c′ = 7.7 (6.7-8.9) *vs* 3-6, and tail with small mucron *vs* pointed (Sturhan & Brzeski, 1991; Chizhov *et al*., 2010); from *D. weischeri* by more slender female (a = 88.8 (72.5-102.5) *vs* 40.6 (35.5-44.8)) and male body (a = 89.7 (71.9-97.9) *vs* 54.3 (50.1-60.4)), female c′ = 7.7 (6.7-8.9) *vs* 3.7 (2.9-4.8) and tail tip bearing small mucron *vs* pointed (Chizhov *et al*., 2010); and from *D. tobaensis* the new species can be differentiated by a longer body of 1881 (1523-2095) *vs* 1005.8 (947-1172) μm and 1875 (1690-2089) *vs* 833 (781-885) μm for female and male, respectively, and a = 88.8 (72.5-102.5) *vs* 72.6 (56-96) and 89.7 (71.9-97.9) *vs* 65.8 (51.7-80) in female and male, respectively (Schneider, 1937). Note: means of *D. tobaensis* are calculated herein based on data provided by Schneider (1937).

Notes on habitat and host plant

Apart from the material collected in December 2012, *D. laurae* sp. n. was collected in the type locality more than ten times from May to December during 2012-2014. Nematodes were collected either from plants growing in shallow water (up to *ca* 0.5 m deep) or from plants washed ashore by storms. No pathological symptoms that could be unambiguously assigned to *D. laurae* sp. n. were present on the host plants. In particular, we did not observe symptoms similar to those described by Zeller (1937) as being caused by *D. tobaensis*.

Molecular characterisation and phylogenetic relationships

ITS rRNA gene

One new sequence with a length of 672 bp was obtained from *D. laurae* sp. n. In total, 130 ITS rRNA gene sequences belonging to the *D. dipsaci* species complex were downloaded from GenBank and included in the preliminary alignment. Seven sequences were subsequently excluded from the analysis because of the possibility that they contained a large number of sequence reading mistakes. The final ITS rRNA alignment contained 123 sequences and, after removal of poorly represented regions at the 3′- and 5′-end, was 565 bp in length. A phylogenetic minimum spanning network, showing the relationships between species of the *D. dipsaci* species complex, is given in Figure 4. *Ditylenchus laurae* sp. n. differed from *D. gigas*, *D. oncogenus* and *Ditylenchus* sp. E in at least 12-13 steps. *Ditylenchus dipsaci* was represented by one main ITS haplotype with 65 identical sequences, a second haplotype with four identical sequences, three haplotypes with two identical sequences, and 17 haplotypes, each comprising a unique sequence. *Ditylenchus gigas* also contained one main ITS haplotype (17 sequences), one haplotype with two identical sequences and four haplotypes, each with a unique sequence. Intraspecific ITS rRNA gene sequence variation for *D. dipsaci* reached 1.4% (8 bp) and for *D. gigas* 0.5% (4 bp). *Ditylenchus laurae* sp. n. differed from all other species by 2.4-6.3% (14-32 bp).

Relationships between species of the *D. dipsaci* complex, as inferred from BI analysis of the ITS rRNA gene sequence alignment with a length of 685 bp, and where each species was represented by a single reference sequence, were not well resolved and are presented in Figure 5.

Partial COI mtDNA gene

Three new sequences with a length of 613 bp were obtained from *D. laurae* sp. n. Fifteen *COI* gene sequences of four species from the *D. dipsaci* complex were included in the alignment. The alignment length was 615 bp. The phylogenetic minimum spanning network showing the relationships between *Ditylenchus* species is given in Figure 6. The haplotypes of *D. laurae* sp. n. differed from *D. gigas* and *Ditylenchus* sp. E by 99-101 and 109-111 changes, respectively. Intraspecific *COI* gene sequence variations for *D. dipsaci* reached 4% (25 bp) and for *D. gigas* 2.9% (18 bp). Haplotype variation within *D. laurae* sp. n. was 0.1-0.5% (1-3 bp). The new species dif-

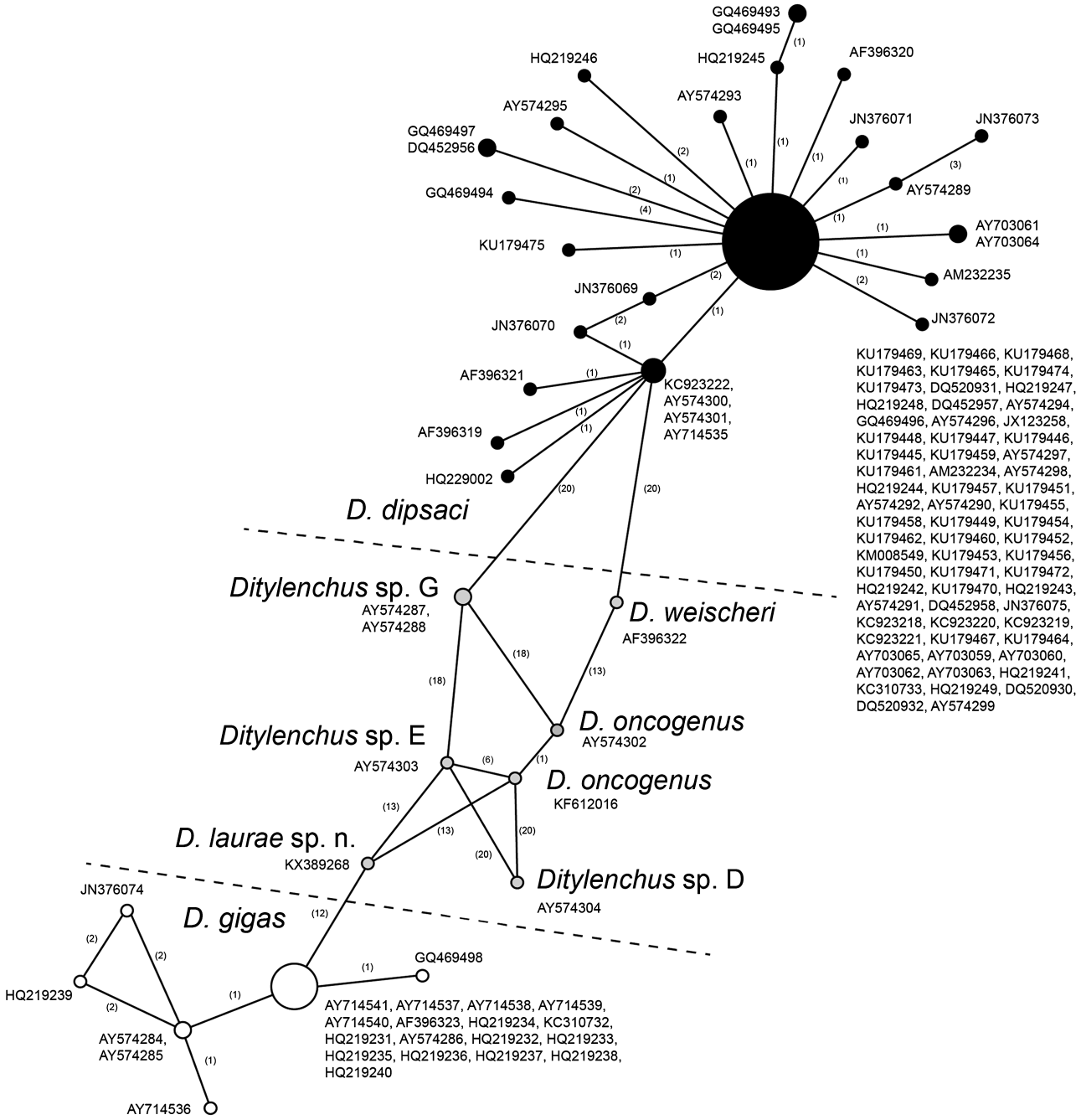

Fig. 4. Phylogenetic minimum spanning network showing the relationships between the ITS rRNA gene sequences of the *Ditylenchus dipsaci* species complex. Sizes of circles are proportional to number of identical sequences. Number of changes between haplotypes are given in brackets along branches.

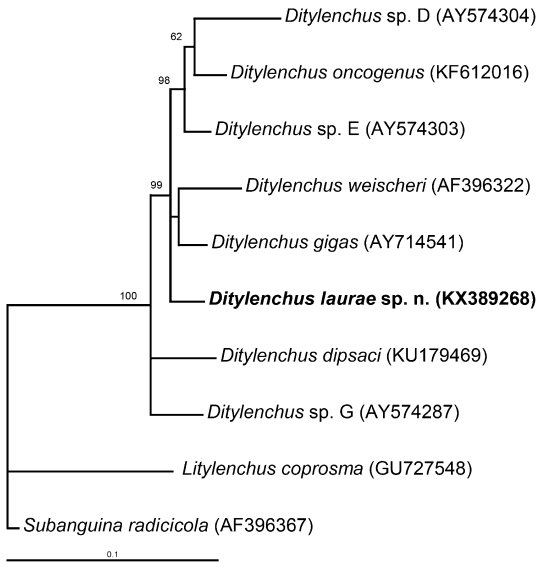

Fig. 5. Phylogenetic relationships between species of the *Ditylenchus dipsaci* complex as inferred from Bayesian analysis of the ITS rRNA gene sequence alignment under the GTR + I + G model. Posterior probability values more than 60% are given for appropriate clades. Newly obtained sequence is indicated in bold.

fered from all other species by 16.4-19.0% (101-115 bp). The phylogenetic relationships of *D. laurae* sp. n. with the other three species from the *D. dipsaci* complex are given in Figure 7.

Partial hsp90 gene

Three new sequences from the *hsp90* clones with a length of 155 bp were obtained from *D. laurae* sp. n. and they differed by 12.9% (20 bp) from each other. Sixteen gene sequences of five species from the *D. dipsaci* complex plus two outgroup sequences were included in the alignment. The alignment length was 247 bp. *Ditylenchus laurae* sp. n. differed from all other species by 11.0-27.2% (19-40 bp). The phylogenetic relationships of *D. laurae* sp. n. with other species from the *D. dipsaci* complex are given in Figure 8.

The *hsp90* sequence analysis revealed a high intraspecific variation for *D. laurae* sp. n. It has been shown that *hsp90* constitutes paralogous gene families that arose by gene duplication events. It seems that our new primers might amplify other paralogous gene in the sample as has been shown, for example, in *Pratylenchus bolivianus* (Troccoli *et al*., 2016), thus compromising the use of this gene fragment as a reliable marker for species delimiting and phylogenetic reconstruction.

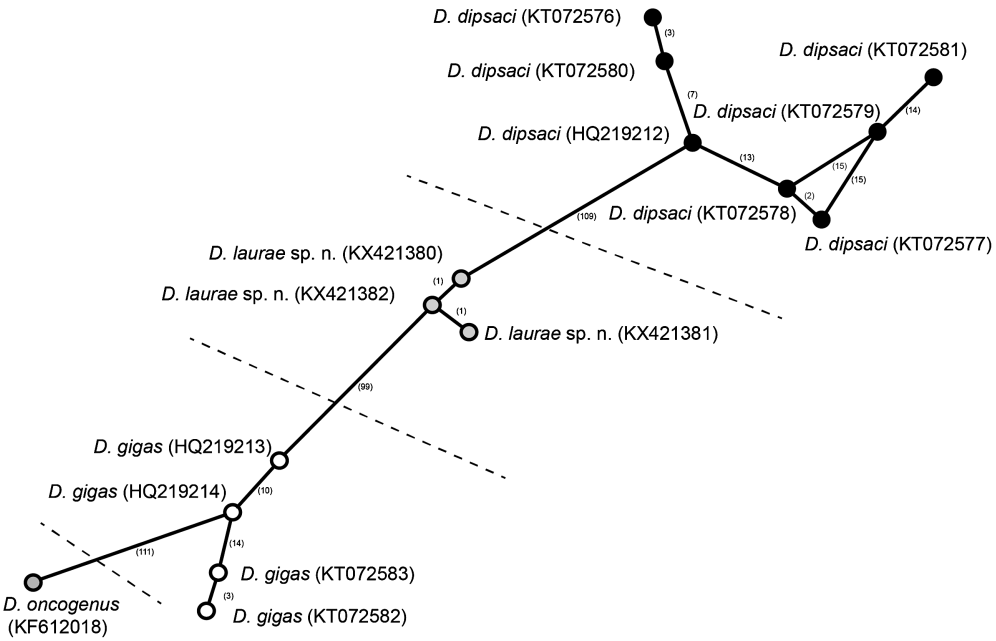

Fig. 6. Phylogenetic minimum spanning network showing the relationships between the *COI* gene sequences of the *Ditylenchus dipsaci* species complex. The number of changes between haplotypes is given in parentheses along branches.

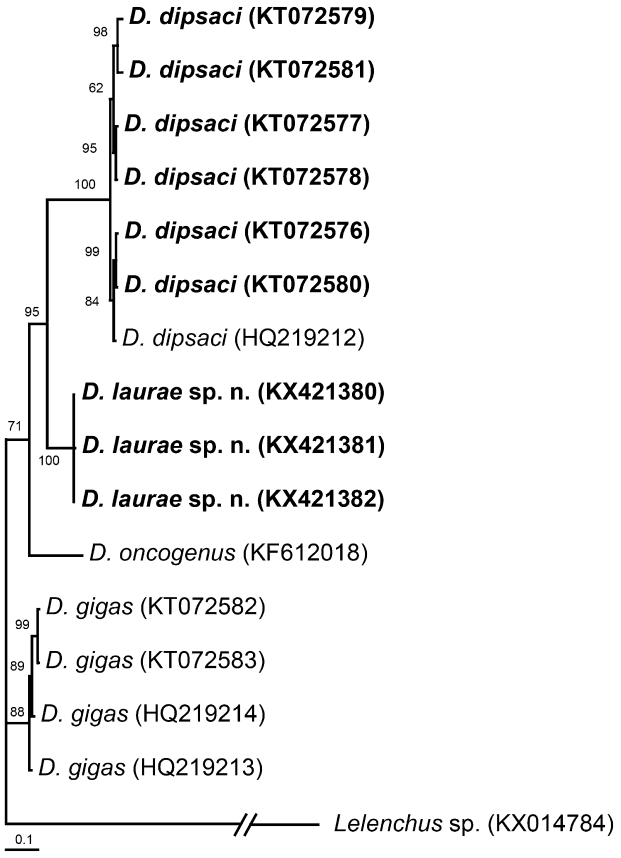

Fig. 7. Phylogenetic relationships between species of the *Ditylenchus dipsaci* complex as inferred from Bayesian analysis of the *COI* gene sequence alignment under the GTR + I + G model. Posterior probability values more than 60% are given for appropriate clades. Newly obtained sequences are indicated in bold.

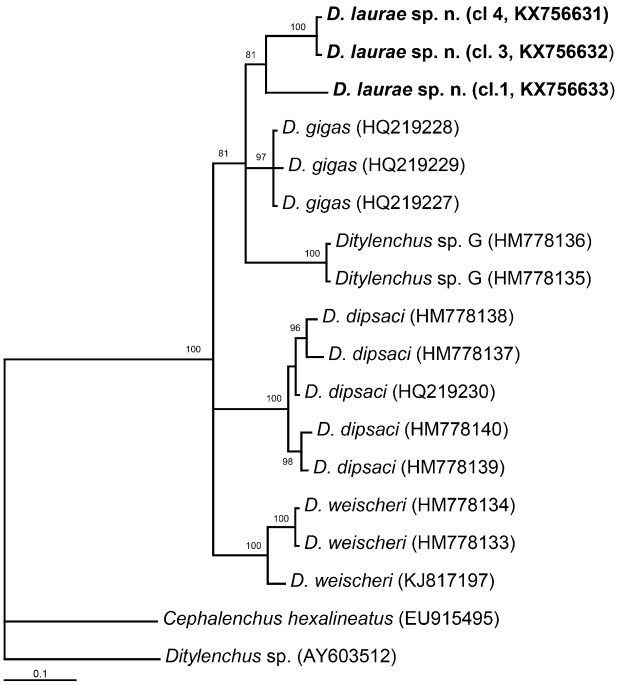

Fig. 8. Phylogenetic relationships between species of the *Ditylenchus dipsaci* complex as inferred from Bayesian analysis of the *hsp90* gene sequence alignment under the GTR + I + G model. Posterior probability values more than 60% are given for appropriate clades. Newly obtained sequences are indicated in bold.

It has been shown that *Ditylenchus* is a paraphyletic taxon that includes several independent lineages (Subbotin *et al*., 2006; Oliveira *et al*., 2013). Our molecular analysis clearly shows that the new species belongs to the *D. dipsaci* complex and is not related to other *Ditylenchus* groups or with *D. angustus* (results not shown), the nematode that causes 'ufra' disease of rice and that also inhabits an aquatic environment (Sturhan & Brzeski, 1991).

The host plant of this species, *P. perfoliatus*, is found in several continents and has a near-cosmopolitan distribution. The pondweed representatives of *Potamogeton*, one of the most important plant genera in the aquatic environment, are especially important as a food resource or habitat for aquatic animals (Haynes, 1974). Studies have also shown some species are important in stabilising substrates, removing particulate matter from the water column, and/or as indicators of water quality (Fritioff & Greger, 2003). The discovery of a new *Ditylenchus* species parasitising *Potamogeton* raises a question as to the possible re-consideration of the status of *D. tobaensis*, a taxon that is currently not considered as valid (see Brzeski, 1991; Sturhan & Brzeski, 1991; Siddiqi, 2000). *Ditylenchus tobaensis* is morphologically similar to *D. laurae* sp. n and parasitises a host from the same genus, possibly representing another valid species of the *D. dipsaci* complex.

At present, we are unable to ascertain any details relating to the biology of *D. laurae* sp. n., including data on dispersal or survival during the winter. The relationships of this nematode with the host plant, for example, which tissues are affected, are also unknown and it is unclear as to what extent this nematode affects this plant, *i.e.*, if it is of major or minor importance to plant health and growth.

Acknowledgements

We thank Mrs Krystyna Gostkiewicz from the National Institute of Agricultural Botany, Cambridge, UK, for specimens of the English population of *D. gigas* used in this study. This research was partially conducted by the Museum and Institute of Zoology, Polish Academy of Sciences, under the project WND-POIG.01.03.01-00-133/09 supported by European Union structural funds through the Operational Programme *Innovative Economy 2007-2013*.

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