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Molecular characterisation and phylogenetic relationships of sedentary nematodes of the genus *Meloinema* Choi & Geraert, 1974 (Nematoda: Tylenchida)

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Summary – Molecular characterisation of two species of *Meloinema*: *M. chitwoodi* from Oregon, USA, and *M. odesanens* from South Korea, is given based on the partial 18S rRNA, the D2-D3 of 28S rRNA, ITS rRNA, and *COI* gene sequences. In the phylogenetic trees, *Meloinema* clustered with *Meloidogyne*, in a basal position and more closely with *Meloidogyne indica* and *M. nataliei*. The Shimodaira-Hasegawa (SH) maximum likelihood testing of an alternative topology with two gene fragments (D2-D3 of 28S rRNA and 18S rRNA genes) did not reject a sister relationship of *Meloidogyne* and *Meloinema*. Molecular results confirmed the view of Siddiqi (2000) that *Meloidogyne* and *Meloinema* evolved from a Pratylenchidae-type ancestor. The clade *Meloinema* + *Meloidogyne* + *Nacobbus* was rejected by the SH test of the D2-D3 of 28S rRNA gene sequence dataset. The molecular results suggested that the genus *Nacobbus* should be placed not in the Meloidogynidae, but in a separate subfamily, the Nacobbinae, under the family Pratylenchidae.

Keywords – *Liriodendron tulipifera*, *Meloinema chitwoodi*, *Meloinema odesanens*, *Nacobbodera*, phylogeny, South Korea, taxonomy, *Tsuga heterophylla*, USA.

The genus *Meloinema* Choi & Geraert, 1974 was first proposed by Choi & Geraert (1974) when they described the type species, *Meloinema kerongense* Choi & Geraert, 1974, from South Korea and placed it in the family Meloidogynidae. Presently, in addition to the type species, four other species have been described: *M. chitwoodi* (Golden & Jensen, 1974) Stone, 1978 from Oregon, USA, *M. maritimum* Eroshenko, 1990 from the Russian Far East, *M. silvicolum* Kleynhans, 1988 from South Africa, and *M. odesanens* Kim, Vovlas, Choi & Lee, 2005 from South Korea. The representatives of this genus are characterised by the presence of vermiform immature females having a vulva near the anus with a bluntly rounded tail, mature females swollen and pear-shaped, sexually dimorphic in the position of the excretory pore, males lacking a bursa and short second-stage juveniles (J2). They have long pharyngeal glands in both sexes and the J2.

Descriptions of two species, *M. kerongense* and *M. chitwoodi*, were published by two author groups with only a few months' difference. *Meloinema chitwoodi* was originally described by Golden & Jensen (1974) as '*Nacobbodera chitwoodi*' using materials collected from Douglas fir in the Coastal Range near Florence and other locations in Oregon, USA. Golden & Jensen (1974) studied this nematode in detail and concluded that certain characters were typical of Nacobbidae Chitwood & Chitwood, 1950 whilst other characters occurred in Heteroderidae Filipjev & Schuurmans Stekhoven, 1941 and Meloidogynidae Skarbilovich, 1959, and they therefore proposed a new genus, *Nacobbodera* Golden & Jensen, 1974, and a new subfamily, Nacobboderinae Golden & Jensen, 1974, within the family Nacobbidae for this new species. After SEM study of males and juveniles, Sher & Bell (1975) found that the face view pattern of *N. chitwoodi* was

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more similar to those of Meloidogynidae or Heteroderidae, rather than to that of *Nacobbus*. These authors, therefore, suggested *Nacobbodera* might be a synonym of *Meloinema*, although specimens of *Meloinema* were not available for study at the time. A few years later, Stone (1978) considered *Nacobbodera* to be a junior subjective synonym of *Meloinema* and transferred *N. chitwoodi* to *Meloinema* in the Meloidogynidae under the subfamily Meloineminae Husain, 1976 (see Husain, 1976). Luc *et al.* (1988) did not accept the synonymisation of *Nacobbodera* with *Meloinema* on the basis of differences between the position of the excretory pore in female and the lengths of the overlapping pharyngeal glands in these genera. Siddiqi (1986, 2000) and Andrássy (2007), however, agreed with the synonymisation and placed these nematodes in the Nacobboderinae under the Meloidogynidae. Bert *et al.* (2006) noticed that, although *Meloinema* was classified within Meloidogynidae, its spermatheca was clearly hoplolaimid-like and lacked the spherical shape with lobe-like protruding cells typical of *Meloidogyne*. Therefore, further phylogenetic analysis of molecular sequence datasets is necessary to clarify the position of *Meloinema*.

During nematological surveys along the Oregon coastal area, USA, in 2019, and the Odae Mountain area of Gwangweon Province, South Korea, in 2004, adults and juveniles of *M. chitwoodi* and *M. odesanens* were extracted from soil samples collected in forests by the first and second authors, respectively, and then preserved and used for molecular study. The goals of the present work were to characterise *Meloinema* species molecularly using rRNA and *COI* genes and to reconstruct the phylogenetic relationships of these species with other tylenchid nematodes.

Materials and methods

Nematode samples and light microscopic study

Nematode populations studied in this research were obtained from soil samples from different locations: *M. chitwoodi* from the rhizosphere of western hemlock, *Tsuga heterophylla* (Raf.) Sarg., from Oregon, USA (Coordinates: 49°29′.141″N, 123°58′.472″W), and *M. odesanens* from a deciduous tree, *Liriodendron tulipifera* L., in the Odae Mountain area of Gwangweon Province, South Korea. Two other nematode species were included in this molecular study: *Pratylenchus* sp. (45°23′.869″N, 123°48′.355″W) from Oregon, USA, and *Nacobbus aberrans* (two samples) from Mexico (Table 1). Nematode specimens were extracted from soil using the centrifugal-flotation method (Jenkins, 1964) and used for morphological identification and molecular study. Light micrographs of live J2 and males of *M. chitwoodi* killed by heating and mounted on temporary slides were taken with an automatic Infinity 2 camera attached to a compound Olympus BX51 microscope equipped with Nomarski differential interference contrast.

DNA extraction, PCR and sequencing

DNA was extracted from several specimens of adults and J2 of each sample using the proteinase K protocol.

Table 1. Nematodes used in the study.

Species	Locality	Host	Sample code	GenBank accession number				Source or reference
				18S rRNA	D2-D3 of 28S rRNA	ITS rRNA	*COI*	
Meloinema chitwoodi	USA, Oregon	*Tsuga heterophylla*	CD3103	MT753454	MT753458	MT753457	MT750293	S.A. Subbotin
M. odesanens	South Korea, Gwangweon Province	*Liriodendron tulipifera*	CA112	MT753453	MT753459	MT753455, MT753456	–	D.G. Kim, Kim *et al.* (2005)
Pratylenchus sp.	USA, Oregon	Unknown plant	CD3108	–	MT753462	–	–	S.A. Subbotin
Nacobbus aberrans sensu lato	Mexico, Saltillo	Unknown plant	CD413	–	MT753460	–	–	I. Cid del Prado Vera
N. aberrans s.l.	Mexico	Unknown plant	CD395	–	MT753461	–	–	I. Cid del Prado Vera

DNA extraction, PCR and cloning protocols were used as described by Tanha Maafi *et al.* (2003) and Subbotin *et al.* (2018). The following primer sets were used for PCR: the forward D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and the reverse D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) primers (Subbotin *et al.*, 2006) for amplification of the D2-D3 expansion segments of 28S rRNA gene; the forward TW81 (5′-GTT TCC GTA GGT GAA CCT GC-3′) and the reverse AB28 (5′-ATA TGC TTA AGT TCA GCG GGT-3′) primers (Tanha Maafi *et al.*, 2003) for amplification of the ITS1-5.8-ITS2 rRNA gene; the forward G18SU (5′-GCT TGT CTC AAA GAT TAA GCC-3′) and the reverse R18Tyl1 (5′-GGT CCA AGA ATT TCA CCT CTC-3′) primers for amplification of the partial 18S rRNA gene; the forward JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) and the reverse JB4 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′) primers (Derycke *et al.*, 2010) for amplification of the partial *COI* gene of mtDNA. PCR products were directly sequenced or cloned and then sequenced. The PCR products were purified using QIAquick (Qiagen) Gel extraction kits and cloned using pGEM-T Vector System II kit (Promega). Sequencing was done at Genewiz. The newly obtained sequences were submitted to the GenBank database under accession numbers: MT753453, MT753454 (18S rRNA), MT753458-MT753462 (D2-D3 of 28S rRNA), MT753455-MT753457 (ITS rRNA) and MT750293 (COI) as indicated in Table 1 and the phylogenetic trees.

Phylogenetic and sequence analysis

The new sequences for the D2-D3 of 28S rRNA and 18S rRNA genes were aligned using ClustalX 1.83 (Chenna *et al.*, 2003) with their corresponding published gene sequences of some tylenchid nematodes (Tigano *et al.*, 2005; Subbotin *et al.*, 2006, 2008; Holterman *et al.*, 2009; Handoo *et al.*, 2014; Álvarez-Ortega *et al.*, 2019 and others). Outgroup taxa were chosen based on previously published data (Subbotin *et al.*, 2006; Holterman *et al.*, 2009). ClustalX with default parameters (gap opening = 15 and gap extension = 6.66) was used to generate for 18S rRNA and D2-D3 of 28S rRNA gene sequence alignments. Additional alignment for the D2-D3 of 28S rRNA gene sequences was generated by this program with the modified parameters (gap opening = 5 and gap extension = 3). Sequence alignments were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) and maximum likelihood (ML) method using PAUP* 4b10 (Swofford, 2003). The best fit model of DNA evolution for ML was obtained using the program jModeltest (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. Two runs were performed for each analysis. The Markov chains were sampled at intervals of 100 generations. After discarding burn-in samples (10%), a 50% majority rule consensus tree was generated. Posterior probabilities (PP) in percentage are given on appropriate clades. Bootstrap support (BS) values for ML trees were calculated by a heuristic search from 1000 replicates.

A Blast search (https://blast.ncbi.nlm.nih.gov/) was used to find a similarity of new sequences with other sequences already deposited in GenBank. Sequence analyses of alignments were performed with PAUP*. Pairwise divergences between taxa were computed as absolute distance values and as percentage mean distance values based on whole alignment, with adjustment for missing data. For testing of alternative topologies in ML, we used the Shimodaira-Hasegawa (SH) test as implemented in PAUP*.

Results and discussion

Morphological identification of *Meloinema* species

The general morphology of specimens of *M. chitwoodi* and *M. odesanens* fitted well with the original descriptions of these species published by Golden & Jensen (1974) and Kim *et al.* (2005), respectively. The J2 and males of *M. chitwoodi* were examined and photographed under light microscopy (Fig. 1). Long pharyngeal glands in the male and J2 (Fig. 1A), absence of a bursa in the male (Fig. 1D), and a conical tail tapering to a rather rounded terminus in the J2 (Fig. 1E) were especially remarkable for *M. chitwoodi*.

Molecular characterisation and phylogenetic relationships

The D2-D3 of 28S rRNA gene

The alignment generated with modified parameters (gap opening = 5 and gap extension = 3) was 800 bp in length and contained 64 sequences. Phylogenetic relationships within selected tylenchid nematodes are given in Figure 2. *Meloinema* species clustered together (PP =

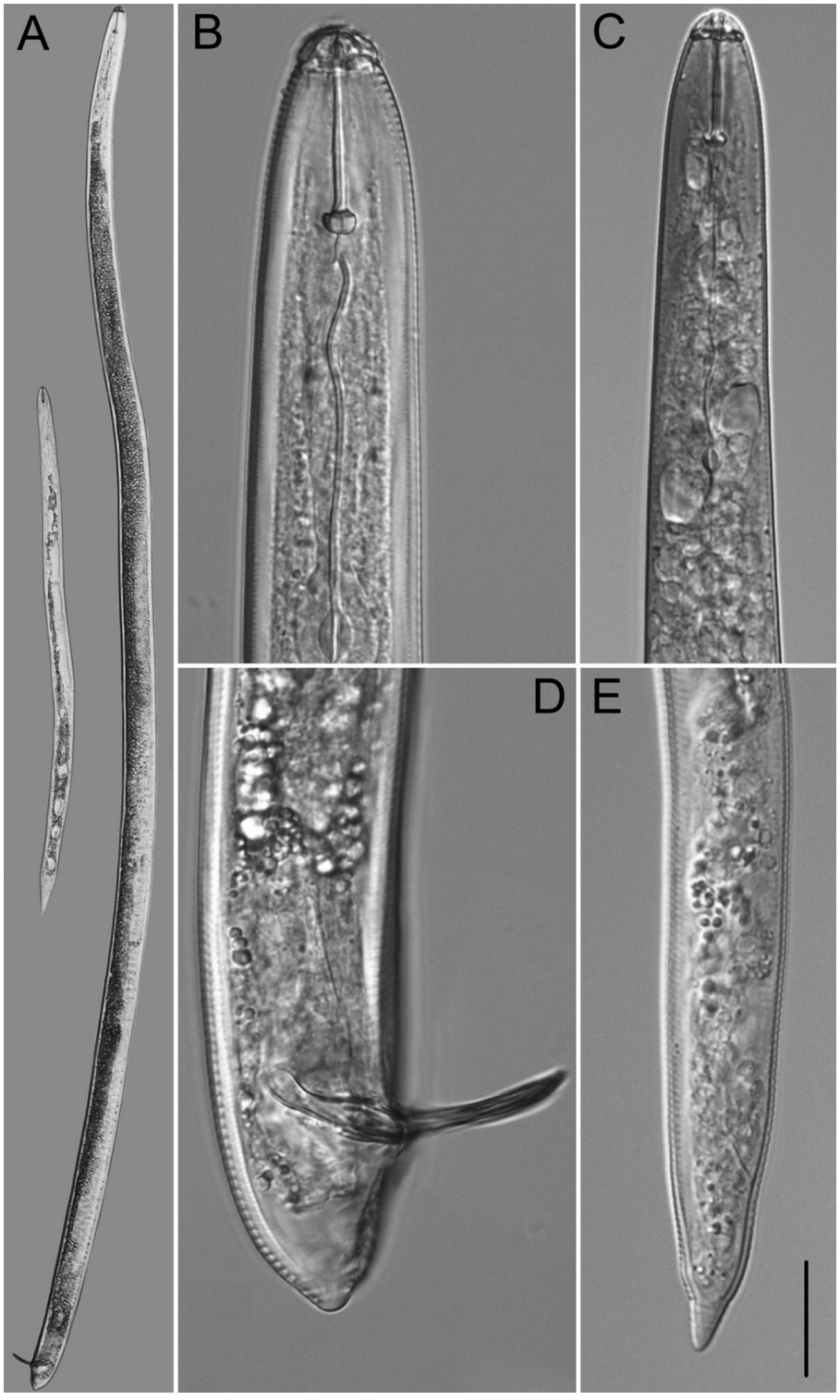

Fig. 1. Photomicrographs of *Meloinema chitwoodi*. A: Entire body of second-stage juvenile (J2) and male (on right); B: Anterior region of male; C: Anterior region of J2; D: Posterior region of male; E: Posterior region of J2. (Scale bar: A = 175 μm; B-E = 25 μm.)

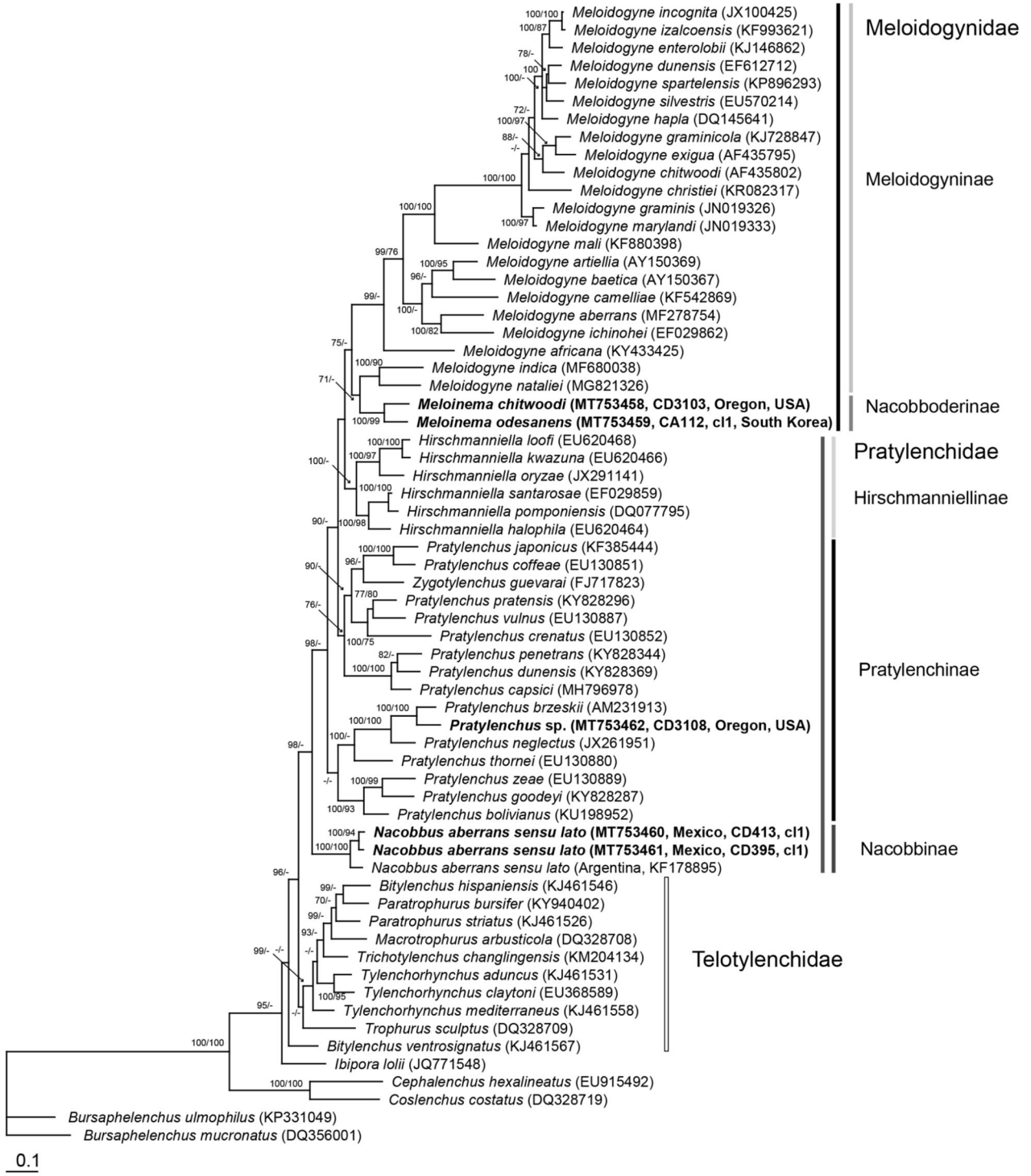

Fig. 2. Phylogenetic relationships of *Meloinema* species with some tylenchid nematodes: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the D2-D3 of 28S rRNA gene sequence alignment (parameters: gap opening – 5 and gap extension – 3) under the GTR + I + G model. Posterior probabilities and bootstrap values equal to, or more than 70% are given for appropriate clades. New sequences are indicated by bold font.

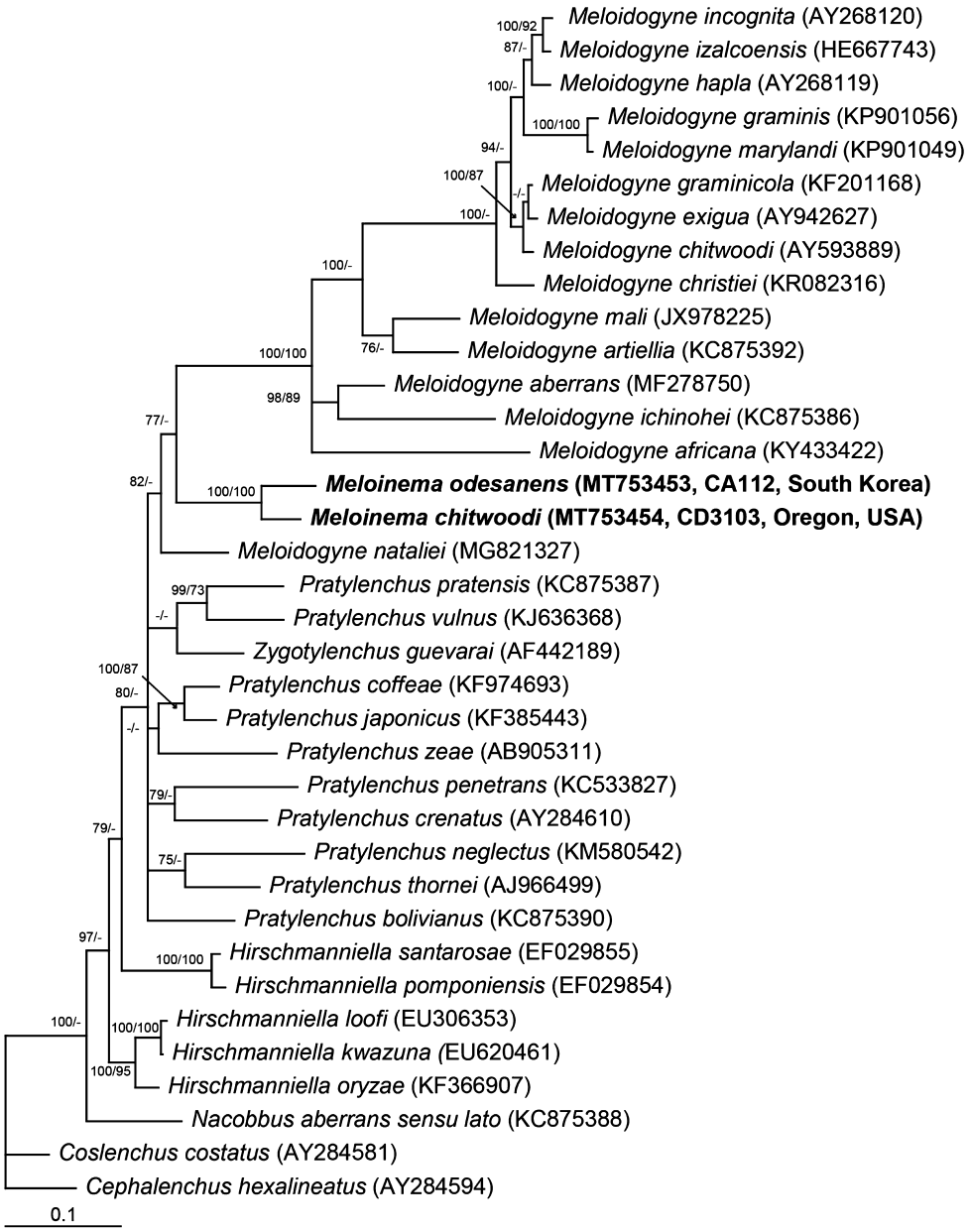

Fig. 3. Phylogenetic relationships of *Meloinema* species with some tylenchid nematodes: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the partial 18S rRNA gene sequence alignment (default parameters) under the GTR + I + G model. Posterior probabilities and bootstrap values equal to, or more than 70% are given for appropriate clades. New sequences are indicated by bold font.

100%, BS = 99%) and formed a clade with *Meloidogyne indica* Whitehead, 1968 and *M. nataliei* Golden, Rose & Bird, 1981 with PP = 71%. The difference between the *M. chitwoodi* and *M. odesanens* gene sequences was 9.8% (67 bp). The alignment generated with default parameters was 745 bp in length. In the BI tree reconstructed from this alignment, *Meloinema* species also clustered together and formed a clade with *Meloidogyne indica* and *M. nataliei* with PP = 98% (not shown).

The 18S rRNA gene

The alignment generated with default parameters was 922 bp in length and contained 36 sequences. Phylogenetic relationships within selected tylenchid nematodes are given in Figure 3. *Meloinema* species clustered together (PP = 100%, BS = 100%) and shared basal positions with *M. nataliei* in the *Meloidogyne* clade. The difference between the *Meloinema chitwoodi* and *M. odesanens* gene sequences was 5.5% (43 bp).

The ITS of rRNA gene

BlastN search of sequences of *Meloinema chitwoodi* and *M. odesanens* in GenBank showed a higher similarity with the ITS rRNA sequences of *Meloidogyne nataliei* (coverage = 49%, identity = 85.23%) and *Pratylenchus mediterraneus* Corbett, 1983 (56%, 81.82%), respectively. The sequence difference between *M. chitwoodi* and *M. odesanens* was 16.0-15.6% (103-105 bp), and the difference between two clones of the ITS-rRNA gene for *M. odesanens* was 4% (30 bp).

COI *mtDNA gene*

BlastN search of sequence of *M. chitwoodi* in GenBank showed the highest similarity with the *COI* sequence of *Meloidogyne ichinohei* Araki, 1992 (coverage = 94%, identity = 87.70%).

Maximum likelihood testing

The results of the Shimodaira-Hasegawa test of tree topologies and alternative phylogenetic hypotheses: *i*) a sister relationship of *Meloidogyne* and *Meloinema*; and *ii*) a single event of sedentary parasitism origin (*Meloidogyne* + *Meloinema* + *Nacobbus* clade) within the Meloidogynidae and Pratylenchidae Thorne, 1949, are given in Table 2. The SH testing of an alternative topology with two gene fragments (D2-D3 of 28S rRNA and 18S rRNA genes) did not reject a sister relationship of *Meloidogyne* and *Meloinema*. A single origin of sedentary parasitism was rejected by SH tests of the D2-D3 of 28S rRNA gene sequence dataset.

In all phylogenetic trees, including the ITS rRNA gene tree (not shown), *Meloinema chitwoodi* and *M. odesanens* are sister species. *Meloinema odesanens* is characterised by long pharyngeal glands largely overlapping the intestine in all developmental stages and an excretory pore positioned consistently midway between the stylet knobs and the median bulb. In the original description of *M. chitwoodi*, the overlapping of the intestine by the gland was less than that in *M. odesanens* and the excretory pore was positioned in the anterior part of the median bulb in both immature the female and juveniles. Luc *et al.* (1988) considered *Nacobbodera* and *Meloinema* as two separate genera based on differences between the position of the excretory pore in the female and the lengths of the overlapping pharyngeal glands. However, these characters are considered as rather variable for these nematodes and cannot be reliable generic characters. Also, the molecular results in this study did not support the placement of these two species in different genera, but rather supported the synonymisation of *Nacobbodera* with *Meloinema*.

In the phylogenetic trees, *Meloinema* tends to cluster with *Meloidogyne* species occupying a basal position. The ML testing does not exclude that these two genera are sister taxa. An interesting result of the present analysis is that sedentary parasitism has independently appeared twice in the Meloidogynidae + Pratylenchidae clade: *i*) the *Meloidogyne* + *Meloinema* lineage; and *ii*) the *Nacobbus* lineage. Thus, molecular results confirmed the view

Table 2. Results of Shimodaira-Hasegawa test of tree topologies and alternative phylogenetic hypotheses.

Hypothesis	Alignment								
	D2 and D3 of 28S rRNA gene (default parameters: 15 and 6.66)			D2 and D3 of 28S rRNA gene (parameters: 5 and 3)			Partial 18S rRNA gene (default parameters: 15 and 6.66)		
	− ln L	Δ ln L	P	− ln L	Δ ln L	P	− ln L	Δ ln L	P
ML tree	17 022.48	best	–	16 292.96	best	–	7572.21	best	–
Meloidogyne and *Meloinema* are sister clades	17 027.44	4.96	0.527	16 293.37	0.41	0.675	7573.05	0.84	0.570
Meloidogyne, *Meloinema* and *Nacobbus* clade	17 073.58	51.10	0.010*	16 314.66	21.69	0.010*	7585.32	13.11	0.129

* Tree significantly worse than the best tree at $P < 0.05$.

of Siddiqi (2000), who suggested that *Meloidogyne* and *Meloinema* evolved from a Pratylenchidae-type ancestor. Our results also confirmed the Siddiqi (2000) view that *Meloidogyne* and *Meloinema* are close relatives, while *Nacobbus* should not be placed in the Meloidogynidae, but as a separate subfamily, the Nacobbinae, under the Pratylenchidae.

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