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Molecular characterisation and phylogenetic relationships of cystoid nematodes of the family Heteroderidae (Nematoda: Tylenchida)

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Summary – The cystoid sedentary nematodes of the Heteroderidae include more than 30 recognised species belonging to from nine to 11 genera, depending on the opinion of the authority. In the present study, we collected nematode populations of species belonging to some of these genera, as well as other cyst-forming species from different locations in the USA, Vietnam, Germany and Russia. The populations of cystoid nematodes represent seven valid species, *Atalodera carolynae*, *Cryphodera sinensis*, *Meloidodera astonei*, *M. floridensis*, *M. mexicana*, *M. sikhotealiniensis*, *Rhizonemella sequoiae*, two unidentified species of *Atalodera*, six unidentified species of *Cryphodera*, and three putative new species of *Rhizonemella*. We also obtained samples of cyst-forming nematodes that include an unidentified species of *Betulodera* from California, and *Heterodera guangdongensis* from Vietnam. A population of *Rotylenchulus* sp. from Arizona, intercepted in Florida, and a population of an unidentified species of *Verutus* from Germany were also added to this study. All of these populations were characterised using sequences of the D2-D3 of 28S rRNA, ITS rRNA or mitochondrial *COI* genes. A total of 89 new sequences were obtained from these analyses. Phylogenetic relationships within the family Heteroderidae were reconstructed based on the D2-D3 of 28S rRNA, ITS rRNA and mitochondrial *COI* gene sequences. Results revealed that the subfamily Ataloderinae was paraphyletic. *Ekphymatodera thomasoni*, a non-cyst-forming species, clustered with the cyst-forming nematodes. Representatives of the subfamily Verutinae formed clades within Heteroderidae. The genus *Meloidodera* was non-monophyletic and distributed within two clades: i) *M. sikhotealiniensis* and *Cryphodera* spp. from Asia and Europe; and ii) *M. astonei*, *M. floridensis* and *M. mexicana* from North America. Based on comparative molecular analysis *Meloidodera alni* syn. n. is proposed as a junior synonym of *M. sikhotealiniensis*. Problems of taxonomy and phylogeography of cystoid nematodes are also discussed.

Keywords – *Atalodera*, *Betulodera*, *COI*, *Cryphodera*, cyst nematodes, D2-D3 of 28S rRNA, *Heterodera guangdongensis*, ITS rRNA, *Meloidodera*, *Meloidodera alni* syn. n., *Meloidodera sikhotealiniensis*, new synonym, *Rhizonemella*, *Rotylenchulus*, *Verutus*.

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Plant-parasitic nematodes of the family Heteroderidae are divided into two major groups: cyst (female body turns into a hard-walled sac or cyst) and cystoid (female body does not turn into a hard-walled cyst) nematodes. Whereas the cyst nematodes are well known pests of many crops and have been studied by many researchers, the cystoid nematodes have received significantly less attention, because of the lesser economic importance of these species in agriculture. Studies of speciation, distribution, diapause and feeding cell formation in hosts for cystoid nematodes may contribute significant knowledge to our understanding of these processes in cyst nematodes. Moreover, understanding evolution of cystoid nematodes is essential for the reconstruction of a natural, stable and predictive taxonomy of the whole family Heteroderidae.

Siddiqi (2000) distinguished nine genera of cystoid nematodes: *Atalodera* Wouts & Sher, 1971, *Bellodera* Wouts, 1985, *Camelodera* Krall, Shagalina & Ivanova, 1988, *Cryphodera* Colbran, 1966, *Ekphymatodera* Baldwin, Bernard & Mundo-Ocampo, 1989, *Hylonema* Luc, Taylor & Cadet, 1978, *Meloidodera* Chitwood, Hannon & Esser, 1956, *Rhizonemella* (Cid del Prado, Lownsbery & Maggenti, 1983) Andr ssy, 2007, and *Sarisodera* Wouts & Sher, 1971. Presently, of 30 recognised species described in this group and listed by Siddiqi (2000), only three cystoid nematode species, *Meloidodera floridensis* Chitwood, Hannon & Esser, 1956, *M. charis* Hooper, 1960, and *M. mexicana* Cid del Prado, 1991 are considered as agricultural pests. In south-eastern USA, *M. floridensis* damages pine trees, especially those grown in nurseries (Inserra & Vovlas, 1986). *Meloidodera mexicana* parasitises cultivated plants such as chilli pepper (*Cap-sicum annuum*), corn (*Zea mays*), tomato (*Solanum lycopersicum*), squash (*Cucurbita pepo*) and beans (*Phaseolus vulgaris*) (Cid del Prado Vera, 1991; Fuentes *et al.*, 1997). This species induced a significant reduction in fresh and dry shoot weight, plant height, root volume, and fruit yield of chilli pepper plants (Fuentes *et al.*, 1997). *Meloidodera charis* was found parasitising corn and okra (*Abelmoschus esculentus*) in Nebraska and Texas and appears to be pathogenic (Heald, 1984; Inserra & Vovlas, 1986; Baldwin & Mundo-Ocampo, 1991).

Several classifications for cystoid nematodes have been proposed and are currently in use. Krall & Krall (1978) included cystoid nematodes in the subfamily Sarisoderinae Krall & Krall, 1978 of the family Heteroderidae Filipjev & Schuurmans Stekhoven, 1941 and in two families, Meloidoderidae Golden, 1971 and Ataloderidae Wouts, 1973. Wouts (1985) distinguished four subfamilies of

cystoid nematodes within the Heteroderidae: Meloidoderinae Golden, 1971, Cryphoderinae Coomans, 1979, Ataloderinae Wouts, 1973, and Verutinae Esser, 1981. Baldwin & Schouest (1990) did not distinguish subfamilies within cyst and cystoid nematodes but recognised the following tribes under Heteroderinae: Heteroderini, Verutini, Meloidoderini, Cryphoderini, Sarisoderini Husain, 1976 and Ataloderini, and proposed four groups based on a phylogenetic analysis of morphological characters: i) cyst nematodes; ii) ataloderins with *Atalodera* and *Camelodera*; iii) sarisoderins with *Sarisodera*, *Rhizonemella*, *Bellodera*, *Hylonema*, and *Ekphymatodera*; and iv) ancestral heteroderids including representatives of *Verutus*, *Meloidodera*, and *Cryphodera* (Baldwin and Mundo-Ocampo, 1991). Siddiqi (2000) placed cystoid nematodes into two subfamilies, Meloidoderinae and Ataloderinae, within the family Heteroderidae, whereas Verutinae was considered to be in the family Rotylenchulidae Husain & Khan, 1967. This classification has been also accepted by Decraemer & Hunt (2013). Andr ssy (2007) also considered cystoid nematodes to be in two subfamilies: Meloidoderinae and Ataloderinae, although he placed *Verutus* and *Bilobodera* in Meloidoderinae.

Phylogenies of cystoid nematodes were reconstructed by several authors using analysis of morphological characters (Krall & Krall, 1978; Coomans, 1979; Ferris, 1979; Wouts, 1985; Baldwin & Schouest, 1990). In recent years new insights into the phylogeny of this group and its relationships with other Heteroderidae have also been proposed using the analysis of rRNA gene sequences (Subbotin *et al.*, 2001, 2006; Ferris *et al.*, 2004; Nguyen *et al.*, 2011; Vovlas *et al.*, 2013; Zhuo *et al.*, 2014a).

In surveys for plant-parasitic nematodes, conducted during the last few years, second-stage juveniles (J2) of unknown cystoid nematodes were detected in several soil samples collected from natural environments in California, Washington, and Florida (USA), as well as in Vietnam, Mexico, Russia and Germany. Some heteroderid juveniles were also identified at the California Department of Food and Agriculture, from regulatory samples shipped from other states in the USA. To identify these nematodes, we conducted a molecular comparative study of these nematode samples with some known cystoid and cyst nematode species described from Europe, Asia, and America. Consequently, the objectives of the present study were to: i) provide molecular characterisation of several valid and unknown species of cystoid nematodes using D2-D3 of 28S rRNA, ITS rRNA or mitochondrial partial *COI* gene sequences; ii) analyse phylogenetic relation-

ships within cystoid nematodes of the family Heteroderidae using these three gene fragments; and *iii*) estimate the species boundaries for cystoid nematode species using an integrated approach.

Materials and methods

NEMATODE POPULATIONS

Nematode populations used in this study were obtained from soil samples collected from different locations in Germany, Mexico, Russia, USA and Vietnam (Table 1). Several females and J2 of *M. sikhotealinensis* Eroshenko, 1978 were identified and provided for this study by A.S. Eroshenko from Russian Primorsky Krai, Russia. *Atalodera carolynae* (Robbins, 1986) Souza & Huang, 1994, *Rhizonemella sequoiae* Cid del Prado Vera, Lownsbery & Maggenti, 1983, and *M. astonei* Cid del Prado Vera & Rowe, 2000 were collected and identified morphologically from their type localities in California, USA, and Mexico, respectively. *Meloidodera floridensis* was collected and identified morphologically from north Florida on *Pinus elliottii*, the same host and locality from where the type specimens were collected and described. One *Rotylenchulus* sp. from Arizona, an unidentified species of *Verutus* from Germany, plus two cyst-forming species were included in this study. Nematode specimens were extracted from soil using the centrifugal-flotation method (Jenkins, 1964) and then frozen (−20°C) for molecular study. Light micrographs were taken of live J2 specimens with an automatic Infinity 2 camera attached to a compound Olympus BX51 microscope equipped with Nomarski differential interference contrast. These micrographs were intended as morphological vouchers of the J2 that were analysed molecularly.

Delimitation of species boundaries for some nematode species used in this study was undertaken using an integrated approach that considered morphological evaluation combined with molecular-based phylogenetic inference (tree-based methods) and sequence analyses (genetic distance methods) (Sites & Marshall, 2004).

DNA EXTRACTION, PCR AND SEQUENCING

DNA was extracted from several specimens of each sample using the proteinase K protocol. DNA extraction, PCR and cloning protocols were used as described by Tanha Maafi *et al.* (2003). 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') primer (Tanha Maafi *et al.*, 2003) or the reverse 5.8SM5 (5'-GGC GCA ATG TGC ATT CGA-3') primer (Nguyen *et al.*, 2011) for amplification of the ITS1-5.8-ITS2 or ITS1 regions; the forward Het-coxiF (5'-TAG TTG ATC GTA ATT TTA ATG G-3') and the reverse Het-coxiR (5'-CCT AAA ACA TAA TGA AA ATG WGC-3') primers (Subbotin, 2015) for amplification of the partial *COI* gene of mtDNA. The following thermal profile was used for *COI* gene amplification: 4 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 45°C and 1 min 30 s at 72°C, with a final extension at 72°C for 10 min. The PCR products were purified using QIAquick (Qiagen) Gel or PCR extraction kits and submitted for direct sequencing or cloned using pGEM-T Vector System II kit (Promega). One to several clones were sequenced from each sample. Sequencing was conducted at Quintara Biosciences. The newly obtained sequences were submitted to the GenBank database under accession numbers: MF425664-MF425752 as indicated in Table 1 and the phylogenetic trees.

PHYLOGENETIC AND SEQUENCE ANALYSIS

The newly obtained sequences for each gene (D2-D3 of 28S rRNA, ITS rRNA and the *COI* mtDNA) were aligned using ClustalX 1.83 (Thompson *et al.*, 1997) with their corresponding published gene sequences (Subbotin *et al.*, 2001, 2006; Ferris *et al.*, 2004; Nguyen *et al.*, 2011; Long *et al.*, 2013; Vovlas *et al.*, 2013; Zhuo *et al.*, 2014a, b; Van den Berg *et al.*, 2016, and others). Alignment program was run with default (gap opening 15.0; gap extension 6.66) parameters for the D2-D3 and *COI* datasets and modified (gap opening 5.0; gap extension 3.33) parameters for the ITS dataset. Four alignments were constructed: *i*) D2-D3 of 28S rRNA gene; *ii*) ITS rRNA gene; *iii*) *COI* gene; *iv*) alignment containing these three gene fragments. Outgroup taxa for each dataset were chosen based on previously published data (Subbotin *et al.*, 2006). Sequence alignments were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) under the GTR + G + I model. BI analysis for each gene was initiated with a random starting tree and was run with four chains for 2.0×10^6 generations. Two runs were performed for each analysis.

Table 1. Species and populations of cystoid and cyst nematodes of the family Heteroderidae, including a species of *Roylenchulus*, used in this study.

Species	Locality	Host	Sample code	GenBank accession no.			Source or reference
				D2–D3 of 28S rRNA	ITS rRNA	COI	
<i>Atalodera carolynae</i>	USA, California, Marin County, Tomales Bay, Keyes Creek	<i>Festuca</i> spp.	CD882, CD891	MF425692, MF425693	MF425714, MF425715	MF425751, MF425752	S.A. Subbotin
<i>Atalodera</i> sp. A	USA (intercepted by CDEA, USA)	Unknown plant	CD1355	MF425694	–	–	S.A. Subbotin
<i>Atalodera</i> sp. B	USA, Washington, Olympic National Park	Unknown plant	CD922	MF425689	–	MF425750	S.A. Subbotin
<i>Atalodera</i> sp. B	USA, Washington, Olympic National Park	Unknown plant	CD946	MF425690	MF425712	MF425749	S.A. Subbotin
<i>Atalodera</i> sp. B	USA, California, Sacramento county, River Bend Park	Unknown plant	CD837	MF425691	MF425713	–	S.A. Subbotin
<i>Cryphodera sinensis</i>	Vietnam, Ninh Binh Province, Cuc Phuong National Park	Wild banana, <i>Musa</i> sp.	1924, CD2230	MF425667	–	–	C.N. Nguyen
<i>C. sinensis</i>	Vietnam, Ninh Binh Province, Cuc Phuong National Park	Forest bamboo, <i>Arundinaria</i> sp.	1800, CD2231	MF425669	–	–	C.N. Nguyen
<i>C. sinensis</i>	Vietnam, Tuyen Quang Province, Na Hang	Fern, <i>Dicksonia</i> sp.	2012, CD2232	MF425666	MF425718	MF425741	C.N. Nguyen
<i>C. sinensis</i>	Vietnam, Ninh Binh Province, Cuc Phuong National Park	Grasses under forest	1794, CD2235	MF425665	–	–	C.N. Nguyen
<i>C. sinensis</i>	Vietnam, Quang Binh Province, Bó Trạch District	Unknown tree	1969, CD2243	MF425668	–	–	C.N. Nguyen
<i>C. sinensis</i>	Vietnam, Ninh Binh Province, Cuc Phuong National Park	Unknown shrub	1798, CD2242	MF425670	MF425719	MF425738	C.N. Nguyen
<i>C. sinensis</i>	Vietnam, Hanoi, Ba Vi National Park	Mimoraceae	2323	MF425664	–	–	Nguyen <i>et al.</i> (2011)
<i>C. sinensis</i>	Vietnam, Ninh Binh Province, Cuc Phuong National Park	Unknown tree	2339	–	JF894395, JF894396	MF425737	Nguyen <i>et al.</i> (2011)
<i>C. sinensis</i>	Vietnam, Ninh Binh Province, Cuc Phuong National Park	Wild banana, <i>Musa</i> sp.	2327	–	JF894389, JF894393	MF425740	Nguyen <i>et al.</i> (2011)
<i>C. sinensis</i>	Vietnam, Ninh Binh Province, Cuc Phuong National Park	Wild banana, <i>Musa</i> sp.	2325	–	JF894388	MF425739	Nguyen <i>et al.</i> (2011)
<i>Cryphodera</i> sp. B	Vietnam, Ninh Binh Province, Cuc Phuong National Park	Unknown tree	2338	MF425671	JF894400, JF894402	MF425743	Nguyen <i>et al.</i> (2011)
<i>Cryphodera</i> sp. C	Vietnam, Hai Phong Province, Cat Ba National Park	Plant from Lauraceae	2348	–	JF894397–JF894399	MF425736	Nguyen <i>et al.</i> (2011)
<i>Cryphodera</i> sp. D	Vietnam, Dong Nai Province, Nam Cát Tiên National Park (11.422°N, 107.428°E)	Forest bamboo	CD2310	MF425672	MF425716, MF425717	MF425744	C. Borkent
<i>Cryphodera</i> sp. E	Vietnam, Hai Phong Province, Cat Ba National Park	Plant from Celastraceae family	2347	MF425676	–	MF425742	D. Sturhan, C.N. Nguyen
<i>Cryphodera</i> sp. F	Vietnam, Ninh Binh Province, Cuc Phuong National Park	Unknown tree	1791, CD2234	MF425675	MF425724	MF425732	C.N. Nguyen

Table 1. (Continued.)

Species	Locality	Host	Sample code	GenBank accession no.			Source or reference
				D2–D3 of 28S rRNA	ITS rRNA	COI	
<i>Cryphodera</i> sp. F	Vietnam, Ninh Binh Province, Cuc Phuong National Park	Unknown tree	1789, CD2238	MF425673	MF425725	MF425731	C.N. Nguyen
<i>Cryphodera</i> sp. F	Vietnam, Ninh Binh Province, Cuc Phuong National Park	Unknown shrub	1795, CD2239	MF425674	MF425723	MF425730	C.N. Nguyen
<i>Cryphodera</i> sp. G	Vietnam, Ninh Binh Province, Cuc Phuong National Park	Unknown tree	1799, CD2233	MF425678	MF425721, MF425722	MF425728	C.N. Nguyen
<i>Meloidodera astonei</i>	Mexico, La Purificacion, Municipio de Texcoco, Mexico State	<i>Solanum rostratum</i>	CD1937	MF425679	MF425702, MF425703	MF425727	I. Cid del Prado Vera
<i>M. floridensis</i>	USA, Florida, Gainesville	<i>Pinus elliotii</i>	CD1957	MF425684	MF425706, MF425707	MF425729	R.N. Inerra
<i>M. mexicana</i>	USA, TX (intercepted by CDFA, USA)	Unknown plant	CD681	MF425681, MF425683	MF425704, MF425705	MF425726	S.A. Subbotin
<i>M. mexicana</i>	USA, TX (intercepted by CDFA, USA)	Chilli pepper	CD238	MF425680, MF425682	–	–	S.A. Subbotin
<i>M. sikhotealinensis</i>	Germany, Bremen	Unknown tree	CD2259	–	–	MF425734	D. Sturhan
(= <i>M. alni</i> syn. n.)							
<i>M. sikhotealinensis</i>	Russia, Primorsky Krai	<i>Betula</i> sp.	374, 377	MF425677	MF425720	MF425733	A.S. Eroshenko
<i>Rhizonemella sequoiae</i>	USA, California, Marin County, Lagunitas Lake Park	<i>Sequoia sempervirens</i>	CD889	MF425699	–	–	S.A. Subbotin
<i>Rhizonemella</i> sp. A	USA, California, Marin County, Lagunitas Lake Park	Unknown tree	CD790, CD875	MF425695, MF425696	MF425708, MF425711	MF425746	S.A. Subbotin
<i>Rhizonemella</i> sp. A	USA, California, Marin County, Lagunitas Lake Park	Unknown tree	CD873	MF425697	MF425709, MF425710	MF425745	S.A. Subbotin
<i>Rhizonemella</i> sp. B	USA, California, Mendocino County, Fort Brag	Unknown tree	CD780	MF425698	–	MF425747	S.A. Subbotin
<i>Rhizonemella</i> sp. C	USA, Santa Cruz County, Mount Hermon	Unknown plant	CD890	MF425700	–	MF425748	J. J. Chitambar
<i>Verutus</i> sp.	Germany	Unknown plant	D47, CD322	MF425685	–	–	D. Sturhan
<i>Rorylenchulus</i> sp.	USA, Arizona (intercepted in Florida, St. Augustine)	Arizona grass	N16–00087, CD2034	MF425701	–	–	R. Inerra
<i>Betulodera</i> sp.	USA, California, Yolo County, Putah Creek	Unknown tree	CD1020	MF425688	–	–	S.A. Subbotin
<i>Heterodera guangdongensis</i>	Vietnam, Lao Cai Province, Van Ban Forest	Unknown shrub	1952, CD2237	MF425686, MF425687	–	MF425735	C.N. Nguyen

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. Sequence analyses of alignments were performed with PAUP* 4b10 (Swofford, 2003). 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.

Results

SPECIES IDENTIFICATION AND DELIMITING

In this study, morphological characters of J2 and adults were available for some species and matched those of their original descriptions. For other species, only the morphological characters of J2 were available. Using an approach integrating available morphological characters and molecular criteria, we distinguished seven valid and eleven putative unknown species of non-cyst-forming nematodes of the family Heteroderidae within the studied samples: *Atalodera carolynae*, *Cryphodera sinensis* Zhuo, Wang, Ye, Peng & Liao, 2014a, *Meloidodera astonei*, *M. floridensis*, *M. mexicana*, *M. sikhotealinensis*, *Rhizonemella sequoiae*, two unidentified species of *Atalodera* (sp. A and sp. B), six unidentified species of *Cryphodera* (sp. B, sp. C, sp. D, sp. E, sp. F and sp. G), three unidentified species of *Rhizonemella* (sp. A, sp. B., sp. C) and one species of *Verutus*. Because of limited numbers of the J2 and absence of adults in some samples we did not identify such populations to a species level. Also, we did not make morphological and morphometric comparison of *Atalodera* sp. A and sp. B, *Cryphodera* spp. B-G, *Rhizonemella* spp. A-C and *Verutus* species with the descriptions of known species of these genera, and consider them herein as putative unidentified species. A *Rotylenchulus* sp. morphologically fit a population of *R. parvus* (Williams, 1960) Sher, 1961, from Arizona as described by Lehman & Inserra (1989). The cyst nematode *Betulodera* sp. was also identified to genus level only. *Heterodera guangdongensis* Zhuo, Wang & Liao, 2014b was identified for the first time from Vietnam (Table 1).

Light microscopic photos of anterior and posteriors regions of J2 for *Atalodera carolynae*, *Meloidodera mexicana*, *M. floridensis*, two putative unidentified species of *Atalodera*, three unidentified species of *Rhizonemella*, and one unidentified species of *Cryphodera* sp. D are

given in Figures 1 and 2. We are aware of the limited diagnostic value of these micrographs for the morphological separation of the illustrated species.

MOLECULAR CHARACTERISATION AND PHYLOGENETIC RELATIONSHIPS

The D2-D3 of 28S rRNA gene

The D2-D3 alignment was 689 bp long and consisted of 90 sequences including *Scutellonema brachyurus* and *Helicotylenchus pseudorobustus* used as outgroups. Thirty-eight new sequences of the D2-D3 expansion fragments of 28S rRNA gene were obtained in the present study. Phylogenetic relationships within Heteroderidae and Rotylenchulidae inferred from the analysis of this partial 28S rRNA gene sequences are presented in Figure 3. The resulting phylogenetic tree contained five highly supported major clades: i) Heteroderinae, containing *Heterodera* species along with representatives of Punctoderinae Krall & Krall, 1978 that were divided in two subclades with species belonging to a: *Globodera*, *Paradolichodera*, *Punctodera*, *Cactodera* and b: *Vittatidera*, *Betulodera*; ii) Ataloderinae containing *Atalodera* and *Rhizonemella* species; iii) Rotylenchulinae Husain & Khan, 1967 containing *Rotylenchulus* species; iv) Meloidoderinae containing *Meloidodera* and *Cryphodera* representatives; and v) Verutinae containing an unidentified species of *Verutus*. The clade I containing cyst nematodes clustered with the clade II with a high support (PP = 100), whereas relationships among other clades remain unresolved.

Monophyly of Heteroderinae was highly supported (PP = 100), whereas the relationships between two subclades of Punctoderinae were unresolved. Monophyly at genus level of *Atalodera*, *Rhizonemella* and *Rotylenchulus* was highly supported (PP = 100) from this tree. The clade containing the subfamily Meloidoderinae consisted of two subclades with representatives defined on geographical locations: i) American subclade (PP = 100) – *Meloidodera floridensis*, *M. mexicana*; and ii) Asian and European subclade (PP = 94) – *Cryphodera* spp. and *M. sikhotealinensis*. Thus, the genus *Meloidodera* was non-monophyletic. The genus *Verutus* remained in a clade separated from that of the Heteroderidae and also from that of the Rotylenchulidae.

Meloidodera sikhotealinensis from the Russian Far East clustered with the sample previously identified as *M. alni* Turkina & Chizhov, 1986 (DQ328706) and their sequences differed by 0.4% (3 bp). *Meloidodera astonei* from Mexico clustered within *M. mexicana* samples

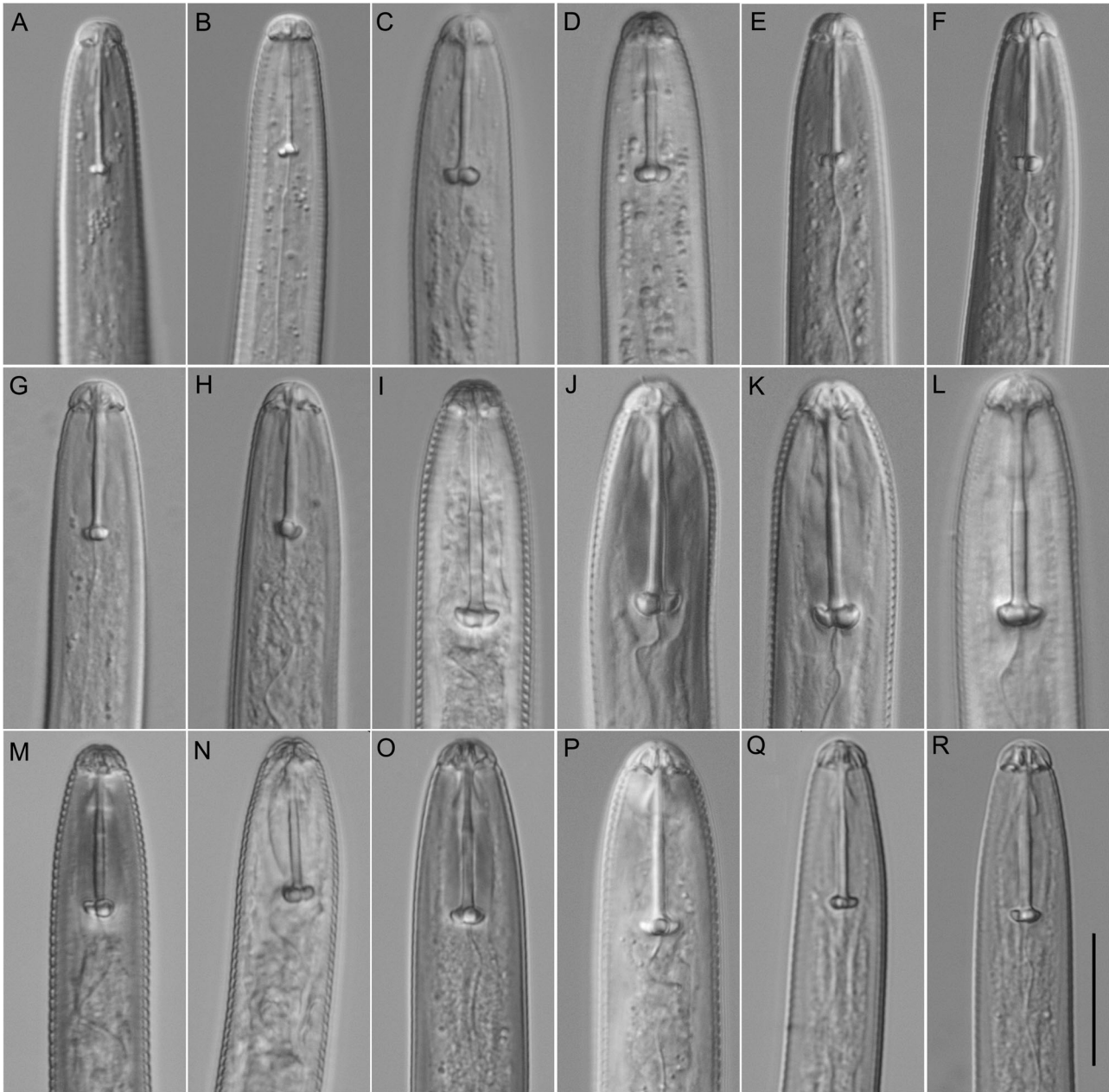


Fig. 1. Photomicrographs of anterior regions of second stage juveniles. A, B: *Atalodera carolynae* (CD882); C, D: *Atalodera* sp. A (CD1355); E, F: *Atalodera* sp. B (CD922); G, H: *Atalodera* sp. B (CD837); I: *Rhizonemella* sp. A (CD875); J, K: *Rhizonemella* sp. B (CD780); L: *Rhizonemella* sp. C (CD890); M, N: *Meloidodera mexicana* (CD238); O, P: *M. floridensis* (CD1957); Q, R: *Cryphodera* sp. D (CD2310). (Scale bar = 20 μ m.)

from the USA, Texas. Intraspecific variations were for: *Cryphodera brinkmani* Karssen & van Aelst, 1999 – 0.9-1.2% (6-8 bp), *C. sinensis* – 0.3-1.0% (3-7 bp), *Rhizonemella* sp. A – 0.4-0.9% (3-6 bp). Interspecific

variation between *A. carolynae* and *A. crassicrustata* was 1.0% (7 bp); *M. floridensis* and *M. mexicana* – 4.3-4.9% (29-33 bp); *Rhizonemella sequoiae* and *Rhizonemella* sp. A – 5.2-5.5% (31-36 bp).

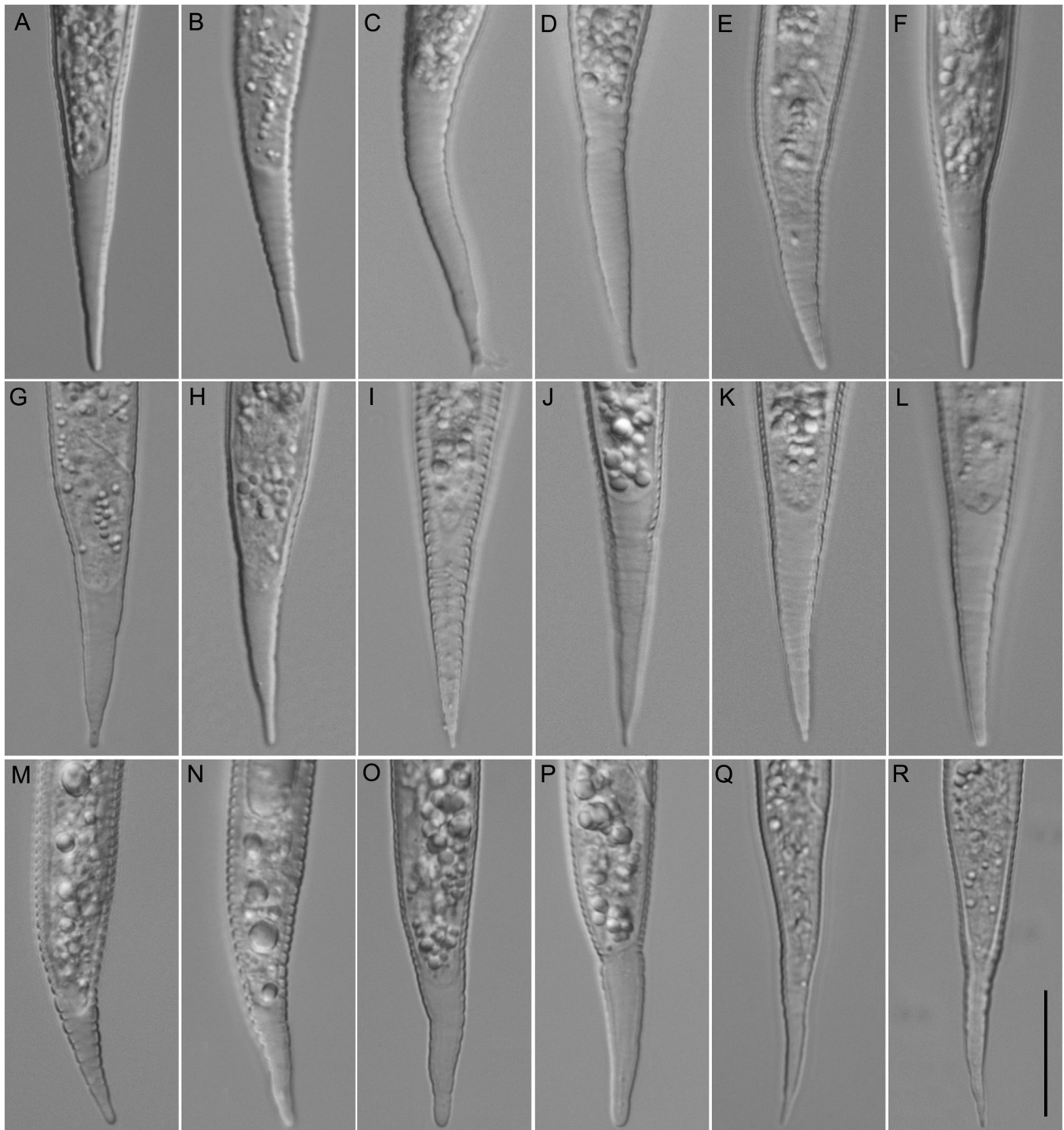


Fig. 2. Photomicrographs of posterior regions of second-stage juveniles. A, B: *Atalodera carolynae* (CD882); C, D: *Atalodera* sp. A (CD1355); E, F: *Atalodera* sp. B (CD922); G, H: *Atalodera* sp. B (CD837); I: *Rhizonemella* sp. A (CD875); J, K: *Rhizonemella* sp. B (CD780); L: *Rhizonemella* sp. C (CD890); M, N: *Meloidodera mexicana* (CD238); O, P: *M. floridensis* (CD1957); Q, R: *Cryphodera* sp. D (CD2310). (Scale bar = 20 μ m.)

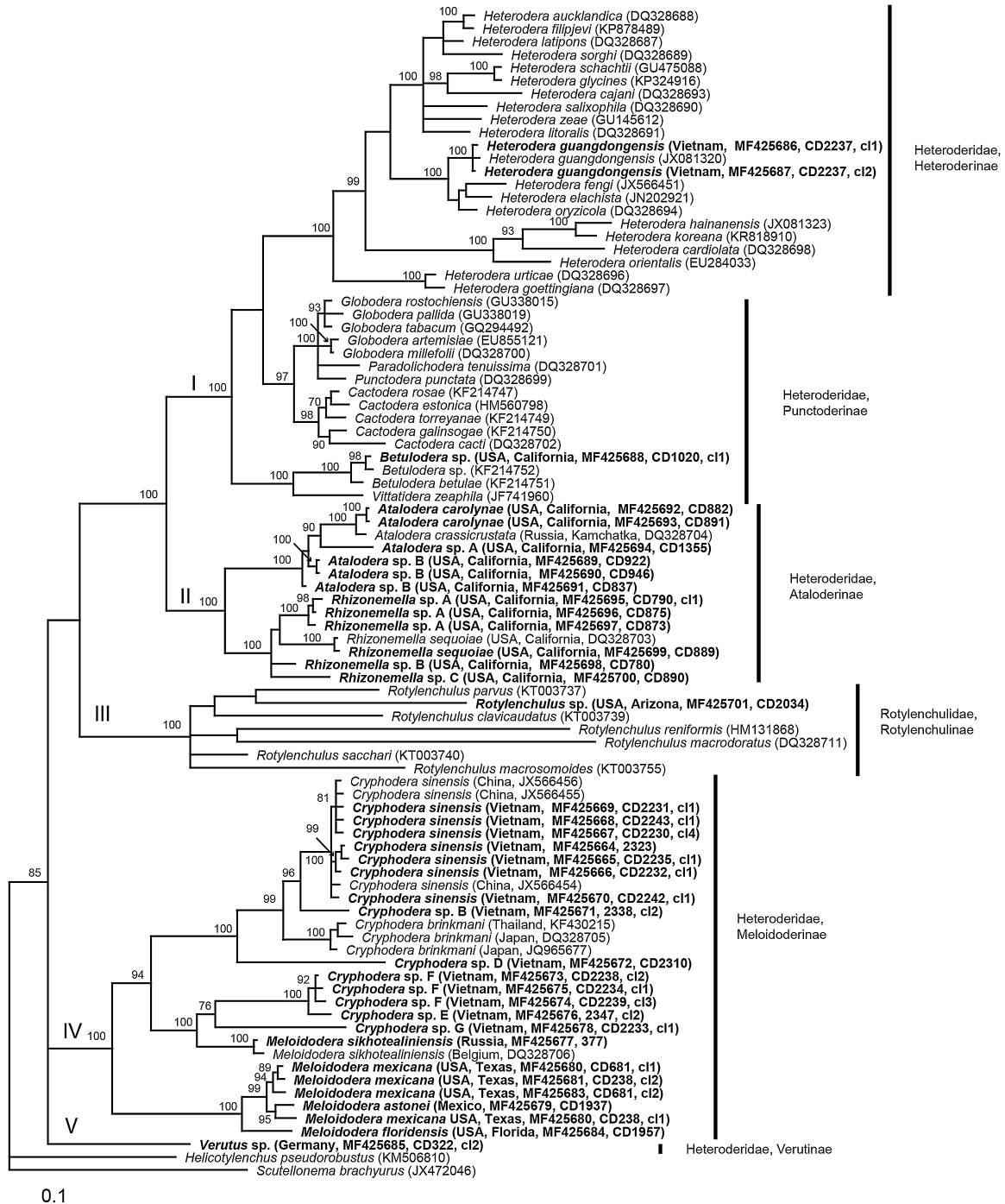


Fig. 3. Phylogenetic relationships within Heteroderidae: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the D2-D3 of 28S rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities equal to, or more than, 70% are given for appropriate clades. Original sequences are indicated by bold font.

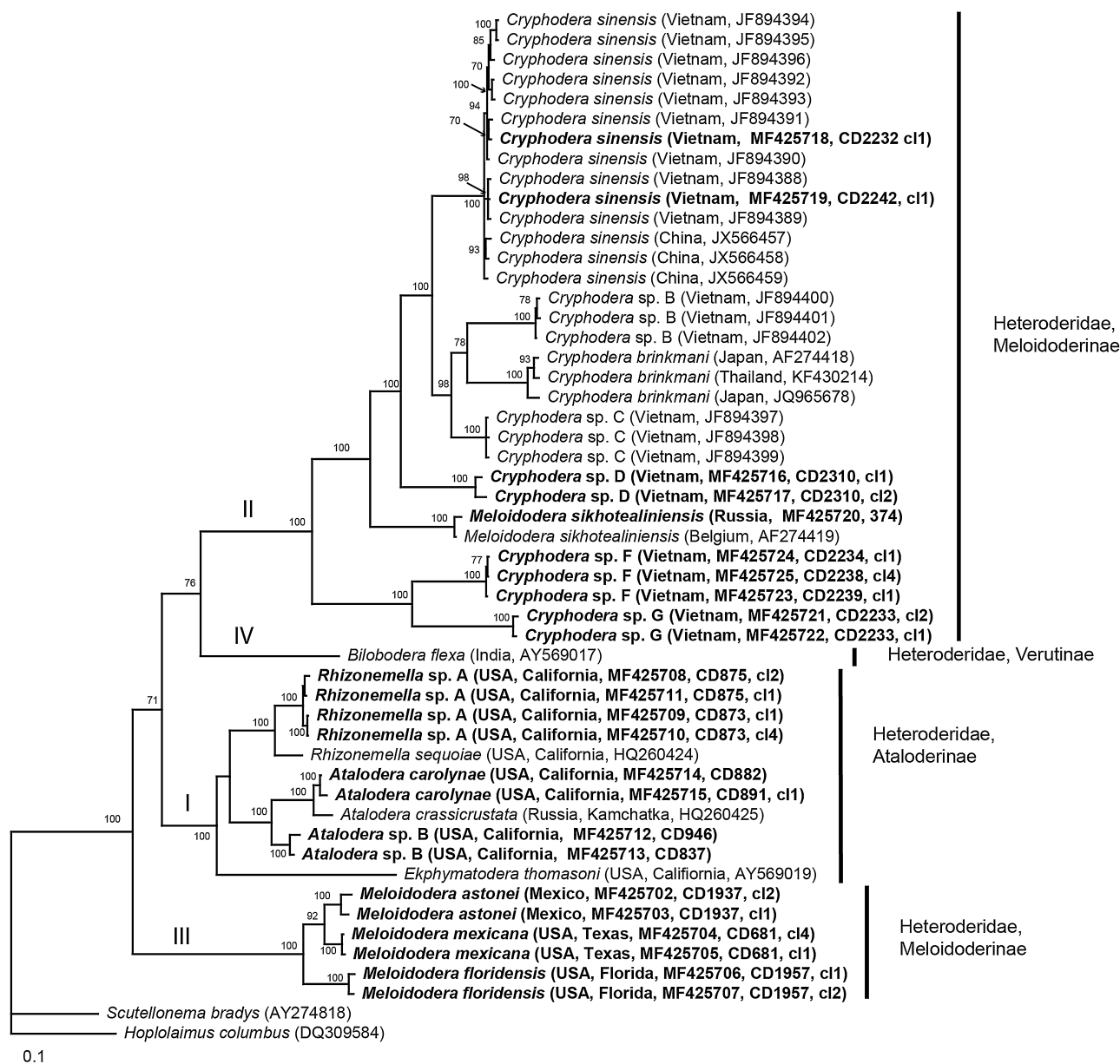


Fig. 4. Phylogenetic relationships within Heteroderidae: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the ITS rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities equal to, or more than, 70% are given for appropriate clades. Original sequences are indicated by bold font.

The ITS1 of the rRNA gene

Because of ambiguity in the ITS2 rRNA alignment, the ITS1 and partial 5.8S rRNA gene sequences were included in the analysis only. The alignment was 872 bp in length and contained 52 sequences. Twenty-four new sequences were obtained for the study. Phylogenetic re-

lationships within Heteroderidae and Rotylenchulidae inferred from the analysis of the ITS rRNA gene sequences are presented in Figure 4. The resulting phylogenetic tree contained four highly supported (PP = 100) major clades: i) Ataloderinae; ii) Asian and European Meloidoderinae; iii) American Meloidoderinae; and iv) *Bilobodera*

(Verutinae), with relationships among the clades unresolved (PP < 80). Monophyly at the genus level of *Atalodera* and *Rhizonemella* was highly supported (PP = 100), whereas the genus *Meloidodera* was paraphyletic.

Meloidodera sikhotealiniensis from the Russian Far East clustered with the sample previously identified as *M. alni* syn. n. from Belgium (AF274419) and their sequences differed by 1.3% (9 bp). *Meloidodera astonei* from Mexico grouped with a *M. mexicana* sample from the USA, Texas and their sequences differed by 7.0-7.8% (39-41 bp). Intraspecific variations were for: *Cryphodera brinkmani* – 1.6-3.6% (11-15 bp), *C. sinensis* – 0.4-2.8% (3-18 bp), *Rhizonemella* sp. A. – 0-1.5% (0-10 bp). Interspecific variation between *A. carolynae* and *A. crassicrustata* was 1% (6 bp), *Rhizonemella sequoiae* and *Rhizonemella* sp. A – 9.7-10% (62-64 bp).

COI mtDNA

The alignment was 472 bp long and contained 32 sequences, including two sequences of *Rotylenchus* used as outgroups. Twenty-seven new sequences of the *COI* gene were obtained in the present study. Phylogenetic relationships within Heteroderidae inferred from the analysis of this partial *COI* gene sequences are presented in Figure 5. The resulting phylogenetic tree contained three highly supported (PP = 92-100) major clades: i) Ataloderinae; ii) Punctoderinae and Heteroderinae; and iii) Meloidoderinae, with relationships among the clades unresolved.

Meloidodera sikhotealiniensis from the Russian Far East clustered with the German *Meloidodera* sample identified as *M. alni* syn. n. and their sequences differed by 6.6% (29 bp). *Meloidodera astonei* from Mexico clustered with a *M. mexicana* sample from Texas, USA, and their sequences differed by 8.1% (38 bp). Intraspecific variations were for *C. sinensis* – 0-2.7% (0-13 bp). Interspecific variation between *Cryphodera brinkmani* and *Cryphodera* sp. C – 11.5% (48 bp); *Rhizonemella* sp. B and *Rhizonemella* sp. C – 10.8% (51 bp).

Combined alignment and condensed tree

The alignment included 36 sequences including three sequences used as outgroups and was 2078 bp in a length. The condensed phylogenetic tree with collapsed branches, with PP less than 80%, is given in Figure 6. The tree consists of six major clades for sedentary nematodes: i) Rotylenchulidae; ii) *Verutus* sp.; iii) *Bilobodera flexa*; iv) *Meloidodera* and *Cryphodera*; v) *Atalodera* and *Rhizonemella*; and vi) *Ekphymatodera thomasoni* and cyst nematodes. The clade *Atalodera* and *Rhizonemella* clustered with the clade *E. thomasoni* and cyst nematodes.

SYNONYMISATION OF *M. ALNI* SYN. N. WITH *M. SIKHOTEALINIENSIS*

Considering the fact that the differences in *COI* and ITS rRNA gene sequences between *M. sikhotealiniensis* and *M. alni* are small and within the range of intraspecific variation, and that the morphological and morphometric characters used to differentiate these species are minor and their host-plant ranges overlap, we propose *M. alni* syn. n. as a junior synonym of *M. sikhotealiniensis*.

Discussion

SPECIES IDENTIFICATION

For the first time in this study, we molecularly characterised *A. carolynae*, *M. astonei*, *M. floridensis*, *M. mexicana* and *Verutus* sp. Our molecular analysis revealed several unidentified and putative new species of *Cryphodera*, *Rhizonemella* and *Atalodera* within the studied samples.

Presently, the genus *Cryphodera* contains seven valid species of which three are found from Asia: *C. kalesari* Bajaj, Walia, Dabur & Bhatti, 1989 (India), *C. brinkmani* (Japan, Thailand) and *C. sinensis* (China, Vietnam), one from Australia: *C. eucalypti* Colbran, 1966 and three from New Zealand: *C. podocarpi* (Wouts, 1973) Luc, Taylor & Cadet, 1978, *C. nothophagi* (Wouts, 1973) Luc, Taylor & Cadet, 1978, and *C. coxi* (Wouts, 1973) Luc, Taylor & Cadet, 1978. After analysis of samples from Vietnam, Nguyen *et al.* (2011) concluded that the morphometrics of J2 cystoid nematodes indicated that at least five *Cryphodera* species might have been present in their studied materials. In our study, we re-analysed the samples from Nguyen *et al.* (2011) and, together with new samples, we identified seven *Cryphodera* species. *Cryphodera sinensis* was identified in most samples and six *Cryphodera* isolates might be considered as putative new species.

Rhizonemella is a genus with only one species, *R. sequoiae*, described by Cid del Prado Vera *et al.* (1983) from coastal redwood *Sequoia sempervirens* in Lagunitas Lake, Marin County, CA, USA. These authors reported that at the same location mature females were also found in smaller numbers on tanbark oak (*Lithocarpus densiflora*), California bay (*Umbellularia californica*), and madrone (*Arbutus menziesii*). It is interesting to note that our molecular analysis revealed a putative new species, *Rhizonemella* sp. A, parasitising an unknown tree located a few hundred metres from the type location of

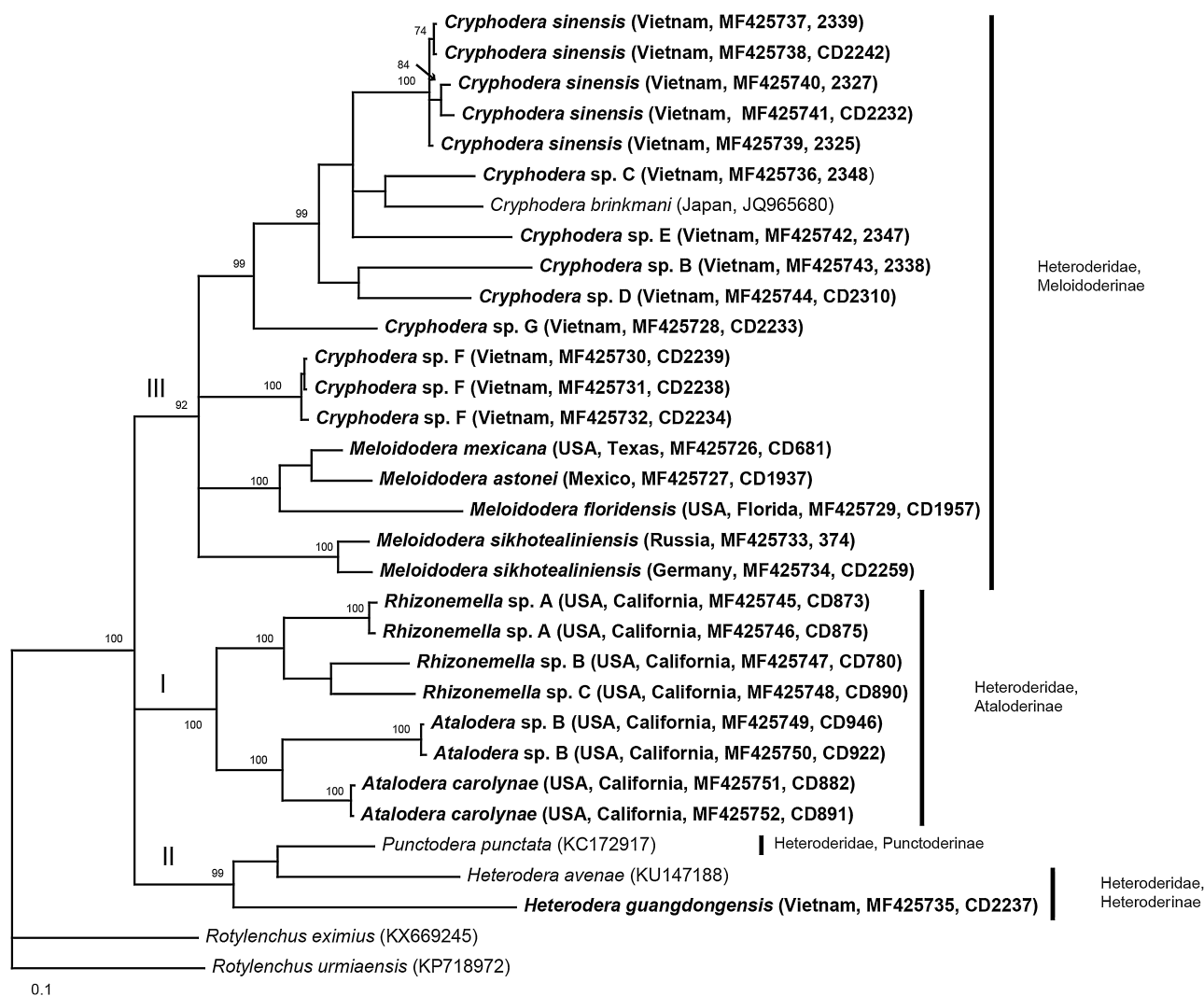


Fig. 5. Phylogenetic relationships within Heteroderidae: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the *COI* mtDNA gene sequence alignment under the GTR + I + G model. Posterior probabilities equal to, or more than, 70% are given for appropriate clades. Original sequences are indicated by bold font.

R. sequoiae. Furthermore, two additional putative new species of *Rhizonemella* (sp. B and sp. C) are also present in Northern California.

Two valid species, *Atalodera crassicrustata* from Kamchatka, Russia, and *A. carolynae*, plus two unidentified species from California, were included in the present study. *Atalodera crassicrustata* and *A. carolynae* are sister taxa and showed only a 1% difference in both rRNA gene sequences, which is within intraspecific variable range for this nematode group. However, these species were distinctly differentiated by morphometric measurements, in-

cluding the J2 stylet length (Bernard, 1981; Eroshenko & Volkova, 2005).

In this study we compared rRNA gene sequences of *M. sikhotealiniensis* with those identified and previously published as *M. alni* syn. n. (Subbotin *et al.*, 2001, 2006). *Meloidodera sikhotealiniensis* was described by Eroshenko (1978) from roots of *Betula mandshurica* in Primorsky Krai of Russia. This species was also found in several other locations in the Russian Far East from *Spiraea salicifolia*, *Ulmus japonica*, *Coryllus* sp., *Populus* sp. and *Chosenia arbutifolia* (Eroshenko, 1978; Eroshenko

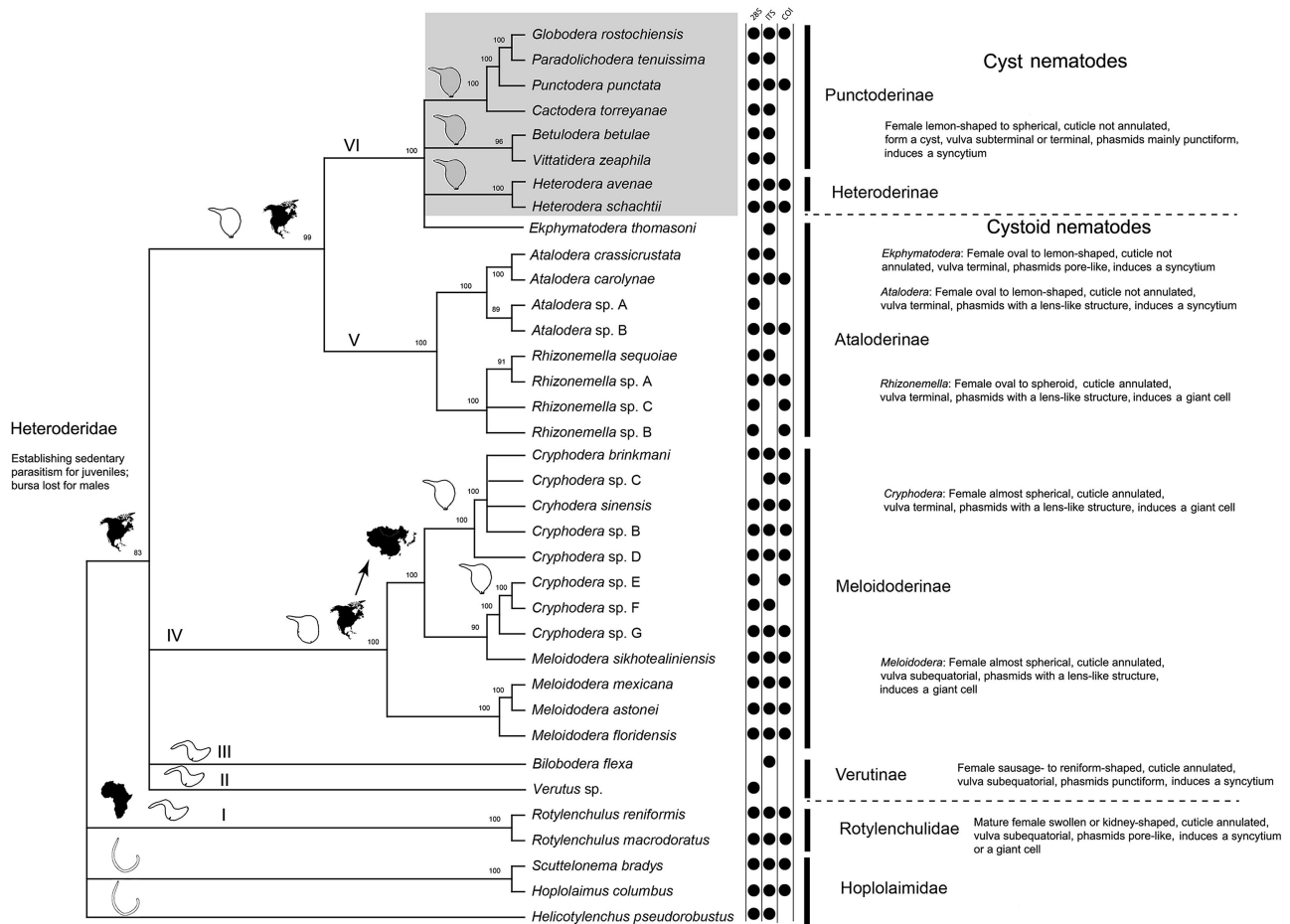


Fig. 6. Phylogenetic relationships within Heteroderidae: Bayesian 50% majority rule consensus condensed tree as inferred from analysis of the sequence alignment containing fragments of three genes under the GTR + I + G model. Posterior probabilities equal to, or more than, 80% are given for appropriate clades. Mapping of geographical regions (Africa, North America and Eastern Asia), female body shape and short taxonomic diagnoses are given for some clades. The gene fragments (partial 28S rRNA, ITS and partial *COI*) included in the dataset for each taxa are also provided.

& Volkova, 2005; Volkova & Kazachenko, 2010). Siddiqi (1986, 2000) regarded *M. sikhotealinensis* as *species inquirenda*. Another species, *M. alni*, was described by Turkina & Chizhov (1986) from *Alnus incana* in the Moscow region, Russia and was later found in many regions of Russia and Europe from *Betula* spp., *Populus* spp. and *Alnus* spp. *Meloidodera alni* was differentiated from *M. sikhotealinensis* by the circular pattern of the vulval region and distance of the dorsal pharyngeal gland orifice from the base of the stylet knobs (DGO) in females, by the location of the hemizonid in males, and by the DGO and ratio of hyaline tail length to tail length in J2 (Turkina & Chizhov, 1986). Considering that these characters have minor taxonomic importance for species differen-

tiation within Heteroderidae, host-plant ranges for these species are overlapped and that the sequence differences between these species are within ranges of intraspecific variations, we propose *M. alni* as a junior synonym of *M. sikhotealinensis*. Thus, the geographic distribution for *M. sikhotealinensis* may be extended from the Russian Far East to Western Europe. A recently reported unidentified *Meloidodera*, as a dominant species in the rhizosphere of poplar and birch trees in Tomsk, Siberia (Antropova & Kartashev, 2016), most likely is *M. sikhotealinensis*.

In the phylogenetic trees, *M. astonei* shows a close relationship with *M. mexicana*. In the D2-D3 of 28S rRNA gene tree, it clustered within *M. mexicana* sequences, perhaps due to ancestral polymorphism of this gene. How-

ever, the ITS and *COI* results show clear separation of these species. *Meloidodera astonei* resembles *M. mexicana* but differs in minor taxonomic morphological characters, including the absence of a capsule covering the female body and shape of the tail and number of annuli in the J2 lip region (Cid del Prado Vera & Rowe, 2000). We retain *M. astonei* as a valid species, although its taxonomic status should be tested further to include additional *Meloidodera* samples. *Meloidodera floridensis* is well separated from the other *Meloidodera* species in all of the trees.

Finally, this study indicates that, despite the morphological similarity of the population of *Rotylenchulus* from Arizona with *R. parvus* (Lehman & Inserra, 1989), it differs from the latter in the rRNA gene sequence.

PHYLOGENY OF HETERODERIDAE

In this study, we provide for the first time a phylogenetic analysis of the subfamilies of the Heteroderidae and Rotylenchulidae. Based on the combined dataset, the subfamily Verutinae, with the two genera *Bilobodera* and *Verutus*, clustered within Heteroderidae, thus reinforcing the view of several authors (Wouts, 1985; Luc *et al.*, 1988; Baldwin & Mundo-Ocampo, 1991; Andr  ssy, 2007) suggesting that Verutinae should be considered within the Heteroderidae and not within the Rotylenchulidae (Siddiqi, 2000; Decraemer & Hunt, 2013).

Molecular analyses showed that Ataloderinae clustered in a sister clade with the cyst nematodes. The condensed tree also showed that *Ekphymatodera thomasoni* strongly clustered with cyst-forming nematodes, thus making the subfamily Ataloderinae paraphyletic. A close relationship of *E. thomasoni* and *B. betulae* was also previously reported by Ferris *et al.* (2004) based on analysis of rDNA sequences. Certain morphological characters of *E. thomasoni* are similar to those found in some cyst nematodes, *e.g.*, fusion of submedial lips with the labial disc, bifid spicules, and pore-like phasmidial openings. After studying the ultrastructure of sperm in *E. thomasoni*, Cares & Baldwin (1994) suggested that the sperm of this species resembled more closely those of cyst-forming taxa than sperm of any other studied cystoid Heteroderidae with respect to numbers of filopodia, presence of cortical microtubules, and fibrous bodies. Baldwin *et al.* (1989) hypothesised that *Ekphymatodera* might be derived from an ancestor intermediate between sarisoderins and ataloderins or *Ekphymatodera* and *Hylonema*, which were monophyletic with cyst nematodes. Baldwin & Schouest (1990) also stated that *Hylonema* and *Ekphymatodera* might rep-

resent a separate tribe, the Hylonemini. The result obtained in our study is based on the analysis of only a single gene for *Ekphymatodera* and therefore additional molecular studies of related taxa need to be conducted with additional genes to confirm the position of this genus within Heteroderidae. We cannot exclude the possibility that the subfamily Ataloderinae might require a revision with perhaps the erection of a new subfamily for *E. thomasoni*.

The phylogenetic analyses confirmed the positions of the genera *Atalodera* and *Rhizonemella*, but not *Cryphodera* in the subfamily Ataloderinae. According to the molecular results, the genus *Cryphodera* should be considered in the subfamily Meloidoderinae. Several authors have emphasised the close relationships between *Meloidodera* and *Cryphodera* (Wouts, 1973; Krall & Krall, 1978). Wouts (1973) placed these genera in the subfamily Meloidoderinae and proposed that *Cryphodera* probably evolved from *Meloidodera* by a shift of the vulva to a terminal position (Wouts & Sher, 1971), thereby distorting the area between vulva and anus with the anus becoming located on a separate elevation slightly outside the body contour of the female. Wouts (1985) also suggested that *Cryphodera* was most closely related to *Meloidodera* from which it differed by the subterminal position of the vulva. Sturhan (2016) revealed that male phasmids appear to be absent in all species of the genera assigned to the subfamilies Heteroderinae and Ataloderinae, but were present and often distinct in the genera *Cryphodera* and *Meloidodera* of the Meloidoderinae. Close relationships among these genera are confirmed by molecular analyses. However, results of our study revealed that *Meloidodera* is non-monophyletic based on both the rRNA genes, and that their representatives were distributed within two clades. The first clade in our trees includes *M. sikhotealinensis* from Asia and Europe and *Cryphodera* spp. found in Asia, and the second clade contains *M. astonei*, *M. floridensis* and *M. mexicana*, which are all species from North America. Variability in the structure of the phasmids and lip region of the J2 and male also led Baldwin (1986) to the conclusion that *Meloidodera* might be polyphyletic. After considering the structure of the male spicule tips and J2 phasmids, Sturhan (pers. comm.) also concluded that this genus might be paraphyletic. Thus, a taxonomic revision of *Meloidodera* is still required to define taxa on the basis of phylogenetic groups inferred from morphology and molecular datasets.

In this study, mapping of a few biological and morphological characters on the molecular phylogenetic tree allows an estimation of evolutionary change. The distribu-

tion of characters in cystoid nematodes, such as the type of feeding cell (a giant cell or syncytium) induced in the host and the expression of female annulation (present or absent), seems to be mosaic, whereas a transformation series of the vulva position from subequatorial to terminal may be more informative in interpreting phenotypic evolution.

PHYLOGEOGRAPHY

North America is most likely the predominant centre of origin for the cystoid nematodes. Of the 11 cystoid nematode genera considered in the present work, the representatives of seven genera: *Atalodera* (seven species), *Bellodera* (one species), *Ekphymatodera* (one species), *Meloidodera* (seven species), *Rhizonemella* (one valid and three undescribed species), *Sarisodera* (one species), and *Verutus* (two species) are found in North America; five genera: *Atalodera* (one species), *Bilobodera* (two species), *Camelodera* (one species), *Cryphodera* (three valid and six unidentified species) and *Meloidodera* (three species) are found in Asia; two genera *Meloidodera* (one species) and *Verutus* (one undescribed species) are found in Europe; one genus *Cryphodera* (four species) is found in Australia and New Zealand; one genus *Atalodera* (two species) is found in South America; and one genus *Hylonema* (one species) is found in Africa. The analysis of species and genetic diversity may suggest local primary centres of diversity and origin for cystoid nematode genera, which coincide with primary biodiversity hotspots of the world: namely Mesoamerica with the Sierra Madre Mountains (Mexico) for *Meloidodera* and the California Floristic Province with the California Coast Ranges, Sierra Nevada and other mountains for *Atalodera* and *Rhizonemella*. The molecular analyses also reveal strong links between North American and Asian taxa: *Atalodera carolynae* from California with *A. crassicrustata* from Kamchatka and North American *Meloidodera* with Asian *Cryphodera*, suggesting an ancient dispersal of cystoid nematodes from North America to Asia. An important secondary centre of origin and diversity for cystoid nematodes may be located in the mountains of northern Vietnam and southern China of the Indo-Burma Biodiversity hotspot. The high level of species diversity of *Cryphodera*, together with the presence of local endemics and several undescribed new heteroderid genera (Nguyen *et al.*, 2011; Sturhan, pers. comm.), may indicate that a relatively rapid speciation rate of this nematode group occurred in this region.

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