Molecular characterisation of the sedentary cystoid nematode, *Mesodolichodera andina* (Golden, Franco, Jatala & Astogaza, 1983) gen. n., comb. n. (Tylenchida: Heteroderidae) parasitising potatoes in the Andes

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Summary. Molecular characterisation of the sedentary cystoid nematode, *Mesodolichodera andina* gen. n., comb. n. parasitising potato roots collected in a potato field located in Pocsi District, Province of Arequipa, Peru is given based on the D2-D3 of 28S rRNA, ITS rRNA, *COI* and *cytb* gene sequences. This nematode was previously known as *Thecavermiculatus andinus*, *Dolichodera andina*, or *Atalodera andina*. *Mesodolichodera andina* gen. n., comb. n. was found in a mixture with *Globodera rostochiensis*. The phylogenetic analysis of rRNA and *COI* genes amplified from females of *M. andina* gen. n., comb. n. showed that this nematode represents an independent evolutionary lineage closely related with cyst-forming nematodes of the genera *Betulodera* and *Vittatidera*. Based on molecular results and analysis of unique morphological characters this nematode was proposed to belong to a new genus of a new subfamily under the family Heteroderidae. *Atalodera* spp. and *Rhizonemella* spp. collected from different locations in Oregon and California, USA were also molecularly characterised with several markers in this study. Phylogenetic relationships within the family Heteroderidae were reconstructed using of rRNA and *COI* gene sequences.

Key words: California, *COI*, cystoid nematode, *cytb*, D2-D3 of 28S rRNA, ITS rRNA, new genus, new subfamily, Oregon, Peru, potato.

In March of 2020, during a nematology survey several white females of a sedentary nematode in a mixture of females and cysts of *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 were isolated from potato (*Solanum tuberosum* L.) roots collected in a potato field located in Pocsi District Province of Arequipa, Peru. The nematode was morphologically identified as *Atalodera andina* (Golden, Franco, Jatala & Astogaza, 1983) de Souza & Huang, 1994. This sedentary nematode was first reported from the roots of several non-solanaceous plants in the vicinity of Lake Titicaca, Department of Puno, the Andes mountains of southern Peru by Jatala *et al.* (1979) who stated that morphological differences exist between the potato cyst nematodes and a sedentary nematode found in this region. These authors noticed that white females of this nematode were essentially spherical with protruding neck, white to yellowish in colour, and could easily be mistaken for potato cyst nematodes. Franco *et al.* (1980) gave additional details about this nematode and updated its host range. Golden *et al.* (1983) described this nematode as a new species, *Thecavermiculatus andinus* Golden, France,

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Fig. 1. Females of *Mesodolichodera andina* gen. n., comb. n. Scale = 0.5 mm.

Jatala & Astogaza, 1983 and placed it in the genus *Thecavermiculatus* Robbins, 1978 of the subfamily Ataloderinae. However, they mentioned that *T. andinus* differed from other *Thecavermiculatus* by the large vulva-anus distance, lace-like cuticular pattern of females and shorter tail of second stage juveniles. They found that *T. andinus* parasitised potato and other plants including *Oxalis tuberosa* Molina, *Medicago polymorpha* L., *Chenopodium quinoa* C.L. Willdenow (Willd.), *Ullucus tuberosus* Caldas, *Capsella bursa-pastoris* (L.) Medik., *Solanum melongena* L., *Malvastrum corornandelianum* (L.) Garcke, and *Lupinus mutabilis* Sweet. This species was also reported from a potato field in Chile. Subsequently, females, second-stage juveniles (J2) and males of this nematode were studied with scanning electron microscopy by Othman *et al.* (1986).

Wouts (1985) suggested that several morphological characters differentiating *T. andinus* from other representatives of Ataloderinae may justify the erection of a new genus for this species. He chose to transfer this species to the genus *Dolichodera* Mulvey & Ebsary, 1978, as *D. andinus*, of the subfamily Punctoderinae. Sturhan *et al.* (2007) noticed that the cuticle of mature females and 'cysts' of *Dolichodera* and *Paradolichodera* Sturhan, Wouts & Subbotin, 2007 were finely striated, at least in part of their body, which might indicate that these genera represented an intermediate group between 'true' cyst-forming taxa and non-cyst-forming Heteroderidae genera. Placement of this nematode in the genus *Dolichodera* was not supported by several researchers (de Souza & Huang, 1994; Siddiqi, 2000; Andrássy, 2007; Sturhan, 2018), who considered this species as a representative of the genus *Atalodera* Wouts & Sher, 1971 to which it has been transferred as *A. andina* after the synonymisation of *Thecavermiculatus* with *Atalodera* (de Souza & Huang, 1994).

The molecular phylogenetic analysis of several genes amplified from females of this species collected in the present study showed that this species does not belong to the genus *Atalodera* or even to the subfamily Ataloderinae. It represents an independent evolutionary lineage closely related with cyst-forming nematodes and based on molecular and morphological characters it belongs to a new genus, *Mesodolichodera*, and is herein described as *Mesodolichodera andina* (Golden, Franco, Jatala & Astogaza, 1983) gen. n., comb. n.

The goals of the present work were: (*i*) to provide morphological diagnosis of the new genus; (*ii*) to molecularly characterise *M. andina* gen. n., comb. n. using rRNA and mtDNA gene sequences;

(*iii*) to molecularly characterise some cystoid nematodes collected in North America; and (*iv*) to reconstruct the phylogenetic relationships of *M. andina* gen. n., comb. n. with other Heteroderidae using several gene sequences.

MATERIAL AND METHODS

Nematode samples and light microscopic study. Nematodes for this research were obtained from soil samples from several locations (Table 1). Females of *Mesodolichodera andina* gen. n., comb. n. and *Globodera rostochiensis* were collected from roots of potato from a field located in Peru, Arequipa, Pocsi District. Soil samples with *Atalodera* spp. and *Rhizonemella* spp. were collected from Oregon and California, USA (Table 1). Nematode specimens were extracted from soil using the centrifugal-flotation method (Jenkins, 1964) and used for morphological and molecular study. Juveniles and females were mounted in temporary slides for identification. Light micrographs of females were taken with 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 juveniles for *Atalodera* spp and *Rhizonemella* spp. and single females of *M. andina* gen. n., comb. n. using the proteinase K protocol. DNA extraction, PCR and cloning protocols were used as described by Subbotin (2021a). 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 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 for amplification of the ITS1-5.8-ITS2 rRNA gene; 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 AAA TGW GC-3') for amplification of the partial *COI* gene, the forward Het-cytbF2 (5'-CAR TAT TTR ATR TTT GAR GT-3') and reverse Het-cytbR3 (5'-ACH ARR AAR TTR ATY TCC TC-3') primers for amplification of the partial *cytb* gene (Subbotin *et al.*, 2020). Sequencing was conducted at Genewiz (CA, USA). The newly obtained sequences were submitted to the GenBank database under accession numbers as indicated in Table 1 and phylogenetic trees.

Phylogenetic and sequence analysis. The new sequences for the rRNA and mtDNA genes were aligned using ClustalX 1.83 (Chenna *et al.*, 2003) with their corresponding published gene sequences of nematodes from the family Heteroderidae (Subbotin *et al.*, 2017, 2018; Kang *et al.*, 2019; Gu *et al.*, 2020). Outgroup taxa were chosen based on previously published data (Subbotin *et al.*, 2017). ClustalX with default parameters (gap opening – 15 and gap extention – 6.66) was used to generate for the D2-D3 of 28S rRNA gene sequence alignment and the modified parameters (gap opening – 5 and gap extention – 3) was used to generate for the ITS rRNA gene sequence alignment. Sequence alignments were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). BI analysis for each gene was initiated with a random starting tree and was run with four chains for 2.0×10^6 generations as described by Subbotin (2021b). For testing of alternative topologies in ML, we used the Kishino-Hasegawa (KH), Shimodaira-Hasegawa (SH) and Shimodaira Approximately Unbiased (AU) tests as implemented in PAUP* 4.0a169 (Swofford, 2003).

A Blast search (https://blast.ncbi.nlm.nih. gov/) was used to find a similarity of new sequences with other sequences already deposited in the 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.

RESULTS AND DISCUSSION

Species identification and delimiting. Using an approach integrating available morphological characters and molecular criteria, we distinguished the following nematodes from the studied samples: potato cyst nematode, *Globodera rostochiensis* and cystoid nematodes, *Mesodolichodera andinus* gen. n., comb. n. (Fig. 1), *Atalodera* sp. C (Fig. 2), *Atalodera* sp. D, *Rhizonemella* sp. D and *Rhizonemella* sp. E.

Morphological study of *Mesodolichodera andina* gen. n., comb. n. A few females and J2 were obtained. Body of young females white, round to oval (Fig. 1). Vulva short and separated from anus. J2 body short, vermiform. Head offset, with several distinct annuli. Tail short, conically tapering to a rounded terminus. Detailed descriptions of different stages for *M. andina* gen. n., comb. n., are given by Golden *et al.* (1983) and Othman *et al.* (1986).

Table 1. *Mesodolichodera andinus* gen. n., comb. n. and other species of the family Heteroderidae used in this study.

Species	Locality	GPS coordinates	Associated host	Sample code	GenBank accession number			
					D2-D3 of 28S rRNA	ITS rRNA	*COI*	*cytb*
Mesodolichodera andinus gen. n., comb. n.	Peru, Department of Arequipa, Pocsi District	S16°30.504'; W071° 21.031'	Potato	CD3201	OK017441-OK017443	OK017438	OK001953-OK001955	OK020185
Atalodera sp. C	USA, Oregon, Lane County	N43°55.855'; W124°06.558'	Forest plants	CD3109	OK017444	OK017436	OK001951	–
Atalodera sp. C	USA, Oregon, Lane County	N43°55.855'; W124°06.558'	Unknown	CD3132	OK017445, OK017446	OK017435	OK001950	–
Atalodera sp. C	USA, Oregon, Douglas County, Gardiner, Dunes Day Use Area	N43°49.996'; W124°09.107'	Pines and grasses	CD3126	OK017448	–	OK001952	–
Atalodera sp. C	USA, Oregon, Coos County	N42°59.392'; W124°26.367'	Unknown	CD3138	OK017447	–	–	–
Atalodera sp. D	USA, California, Humboldt County, Trinidad, Dry Lagoon State Park	N41°13.228'; W124°06.084'	Unknown	CD3139	OK017449	OK017437	–	–
Globodera rostochiensis	Peru, Department of Arequipa, Pocsi District	S16°30.504'; W071° 21.031'	Potato	CD3201cyst	OK017440	OK017439	OK001957	–
Rhizonemella sp. D	USA, Oregon, Lincoln County, Beverly Beach State Park	N44°43.698'; W124°03.425'	Grasses	CD3125	OK017451	–	OK001956	–
Rhizonemella sp. E	USA, California, Madera County	N37°24.596'; W119°37.329'	Forest plants	CD3473	OK017450	–	–	–

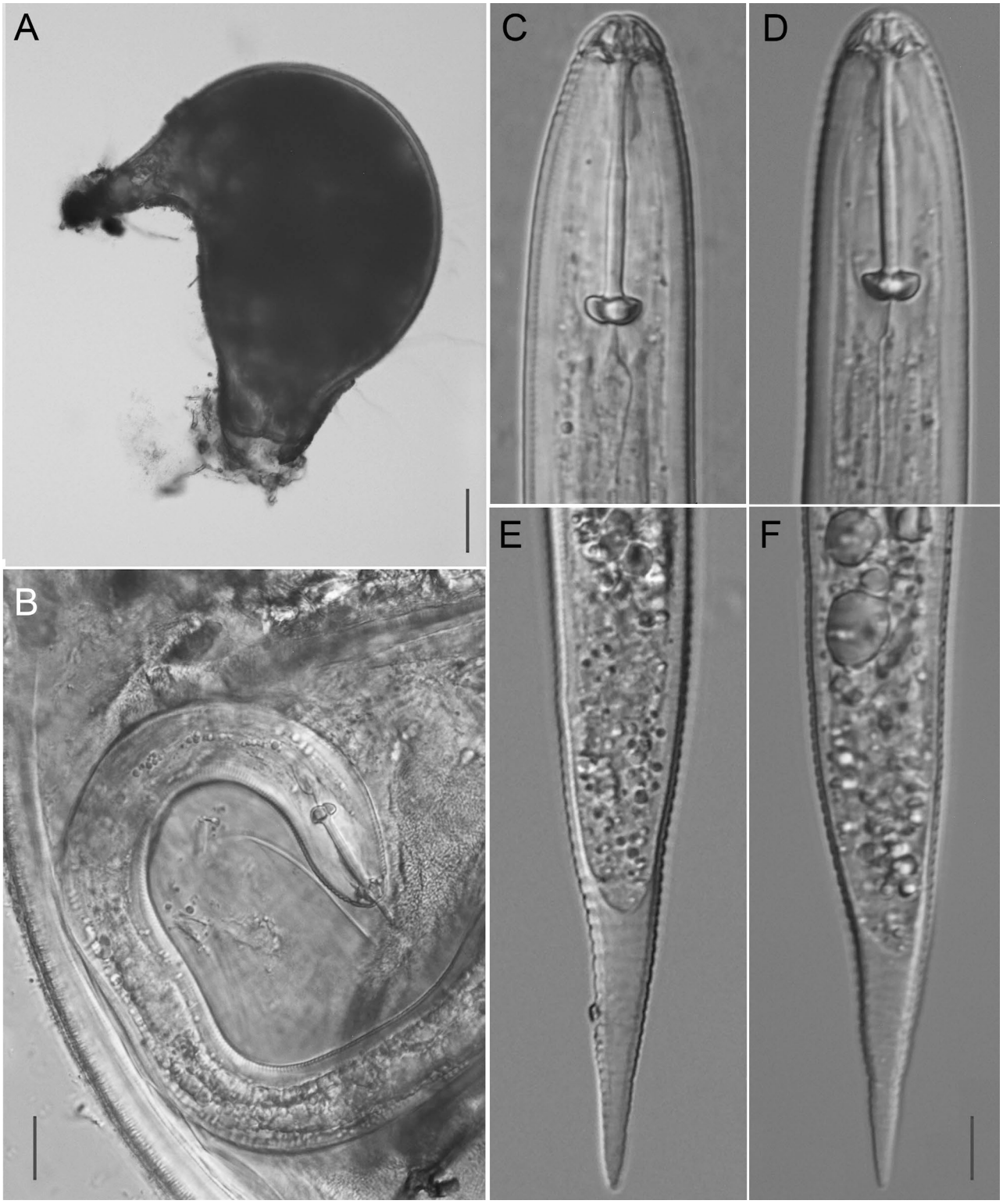

Fig. 2. Sedentary cystoid nematode, *Atalodera* sp. C. (CD3132). A: Female; B: Juvenile inside female body; C & D: Anterior end of second-stage juveniles; E & F: Posterior end of second-stage juveniles. Scale: A = 100 µm; B = 10 µm; C-F = 5 µm.

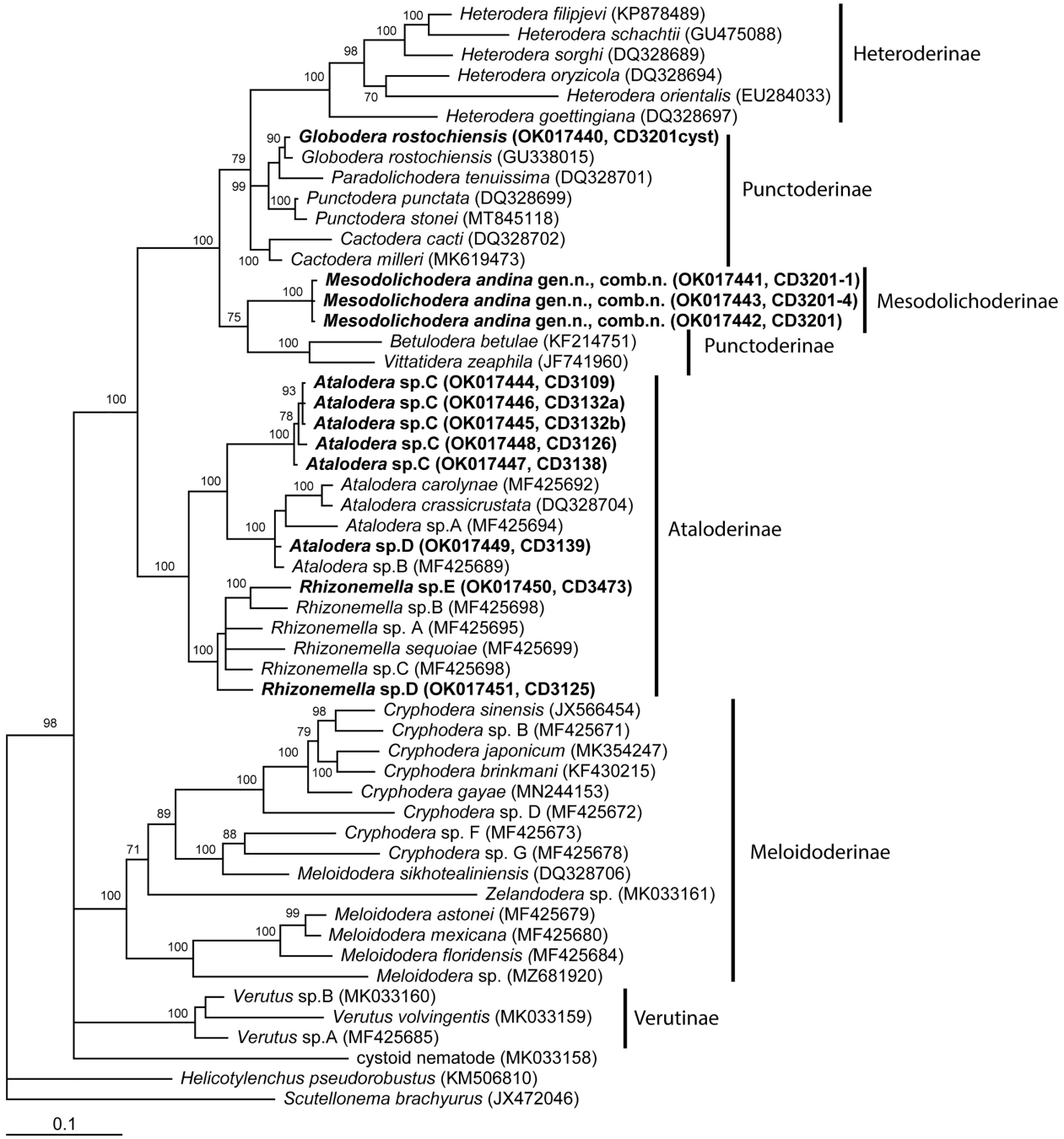

Fig. 3. Phylogenetic relationships of *Mesodolichodera andina* gen. n., comb. n. with other Heteroderidae: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the D2-D3 of 28S rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities and bootstrap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

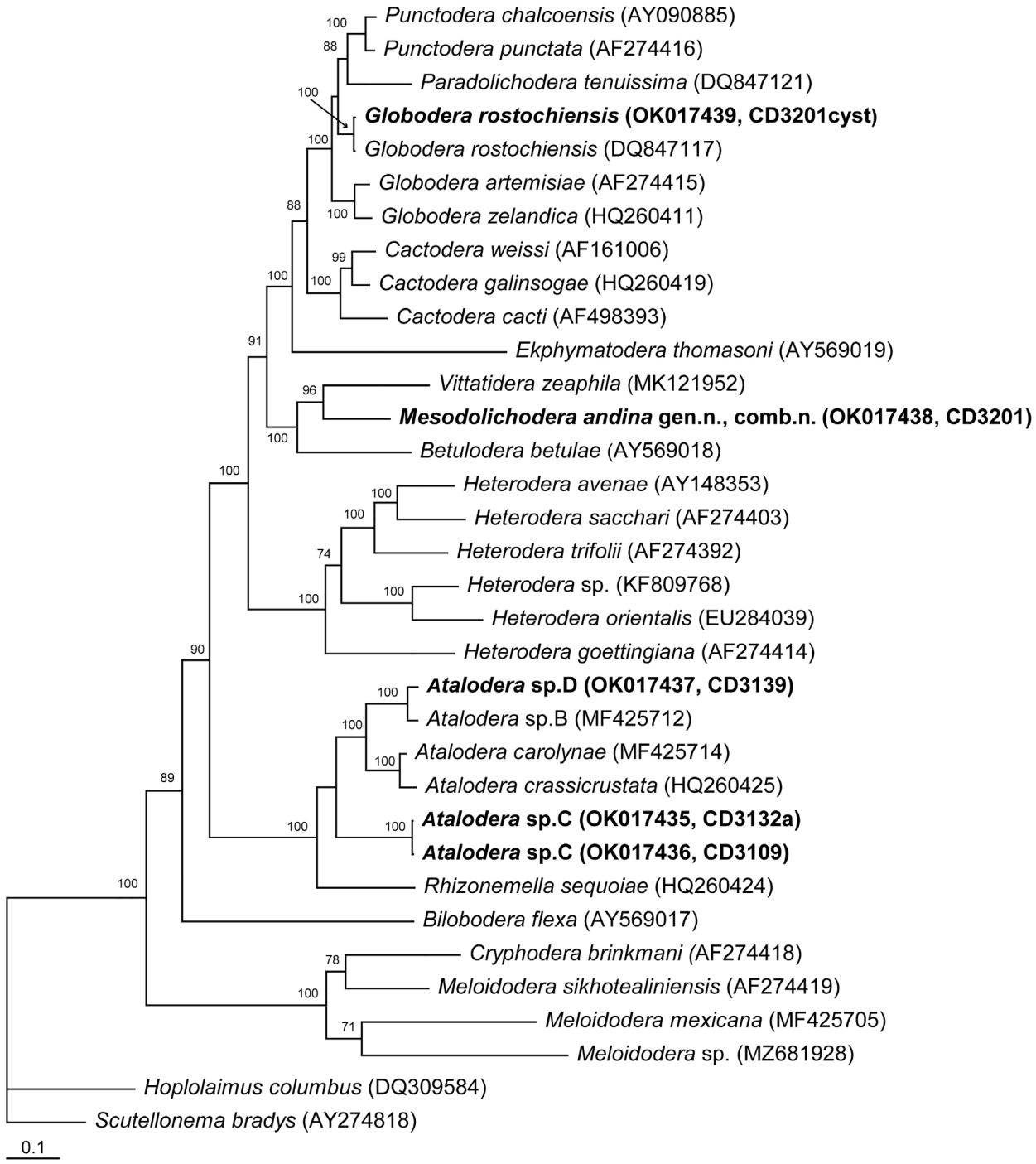

Fig. 4. Phylogenetic relationships of *Mesodolichodera andina* gen. n., comb. n. with other Heteroderidae: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the ITS rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities and bootstrap values equal to, or more than 70% are given for appropriate clades. New sequences are indicated by bold font.

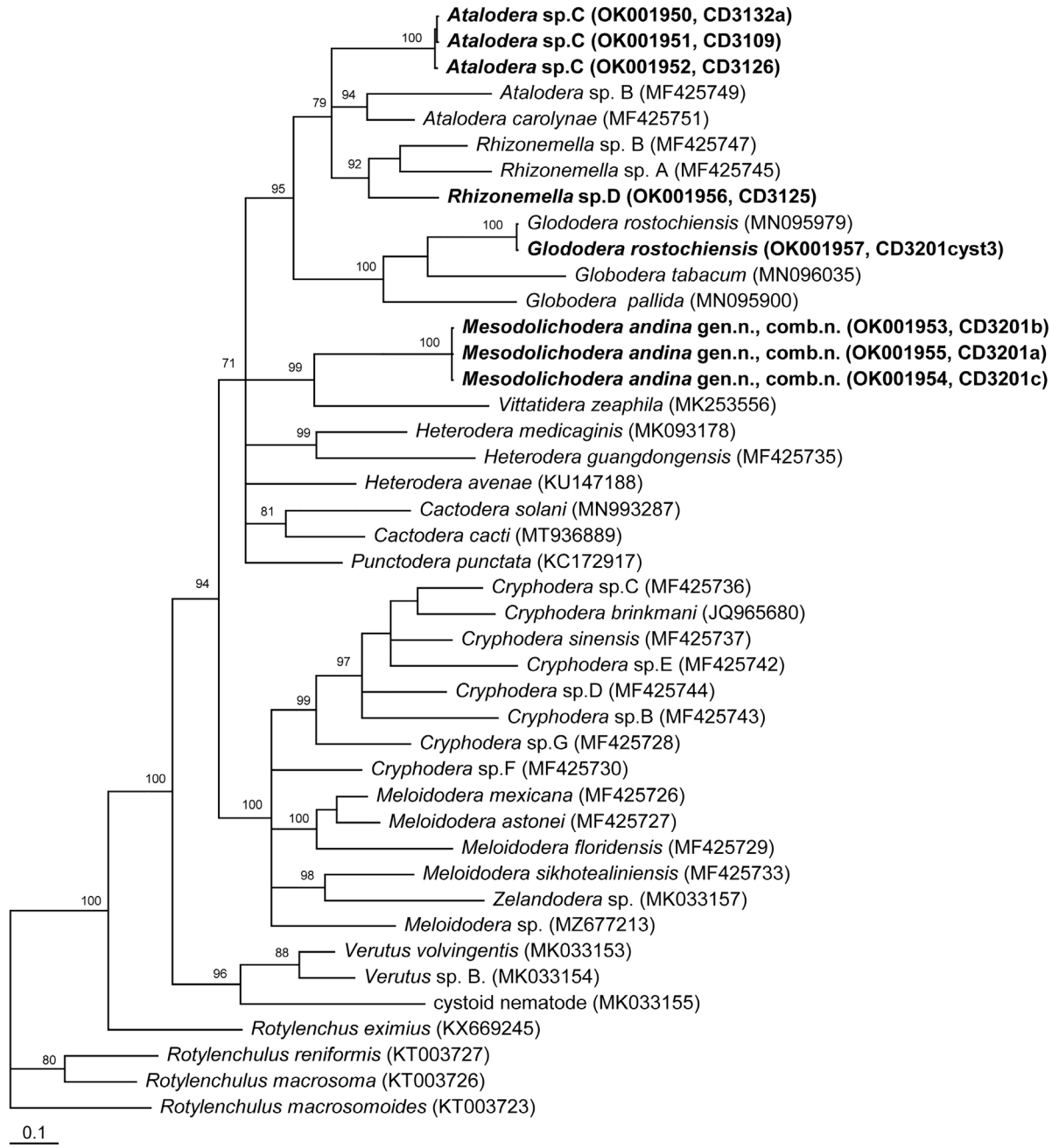

Fig. 5. Phylogenetic relationships of *Mesodolichodera andina* gen. n., comb. n. with other Heteroderidae: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the *COI* gene sequence alignment under the GTR + I + G model. Posterior probabilities and bootstrap values equal to, or more than 70% are given for appropriate clades. New sequences are indicated by bold font.

Molecular characterisation and phylogenetic relationships. The D2-D3 expansion segments of 28S rRNA gene. The alignment contained 52 sequences of the representatives from Heteroderidae and two sequences of the outgoup taxa and it had 695 bp in a length. Twelve new sequences were obtained in this study. Phylogenetic relationships of *M. andina* gen. n., comb. n. with other Heteroderidae are given in Figure 3. The D2-D3 of 28S rRNA gene sequence of *M. andina* gen. n., comb. n. formed a clade (Posterior probability (PP) = 75%); with those of cyst nematodes, *Betulodera betulae* Sturhan, 2002 and *Vittatidera zeaphila* Bernard, Handoo, Powers, Donald & Heinz, 2010

Table 2. Results of tree topology tests and alternative phylogenetic hypotheses.

Alignment / Hypothesis	-ln L	Diff -ln L	KH	SH	AU
	D2-D3 of 28S rRNA gene (parameters: 15 and 6.66)				
ML tree	7824.38	best	–	–	–
Mesodolichodera gen. n. and Punctoderinae are sister clades	7833.49	8.95	0.353	0.378	0.209
Mesodolichodera gen. n. and cyst nematodes (Heteroderinae + Punctoderinae) are sister clades	7828.12	3.59	0.289	0.666	0.188
Mesodolichodera gen. n. clustered with Ataloderinae	7850.09	25.55	0.060	0.086	0.084
	ITS rRNA gene (parameters: 5 and 3)				
ML tree	16273.57	best	–	–	–
Mesodolichodera gen. n. and Punctoderinae are sister clades	16302.49	28.92	0.017*	0.027*	0.004*
Mesodolichodera gen. n. and cyst nematodes (Heteroderinae + Punctoderinae) are sister clades	16309.42	35.84	0.006*	0.011*	0.000*
Mesodolichodera gen. n. clustered with Ataloderinae	16304.08	30.51	0.013*	0.030*	0.012*
	***COI* gene (parameters: 15 and 6.66)**				
ML tree	6827.60	best	–	–	–
Mesodolichodera gen. n. and Punctoderinae are sister clades	6842.70	15.10	0.115	0.103	0.120
Mesodolichodera gen. n. and cyst nematodes (Heteroderinae + Punctoderinae) are sister clades	6839.54	11.94	0.265	0.192	0.086
Mesodolichodera gen. n. clustered with Ataloderinae	6836.74	9.14	0.413	0.275	0.344

KH: Kishino-Hasegawa test using RELL bootstrap (two-tailed test); SH: Shimodaira-Hasegawa test using RELL bootstrap (one-tailed test); AU: Shimodaira Approximately Unbiased test.

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

and differed from them in 60 bp and 57 bp (9.1% and 8.7%), respectively. Sequence of *Atalodera* sp. D differed from that *Atalodera* sp. B in 5 bp (0.7%) and the interspecific sequence variation within *Atalodera* sp. C was up to 4 bp (0.6%).

The ITS of rRNA gene. The alignment contained 32 sequences of the representatives from Heteroderidae and two sequences of the outgoup taxa and it had 1319 bp in a length. Five new sequences were obtained in this study. Phylogenetic relationships of *M. andina* gen. n., comb. n. with other Heteroderidae are given in Figure 4. The ITS rRNA gene sequence of *M. andina* gen. n., comb. n. formed a clade (PP = 96%) with that of cyst nematode, *Vittatidera zeaphila* and differed from it in 170 bp (19.0%). Sequence of *Atalodera* sp. D differed from that of *Atalodera* sp. B in 27 bp (3.0%).

The partial *COI* mtDNA gene. The alignment contained 39 sequences of the representatives from Heteroderidae and four of the outgoup taxa and it had 447 bp in a length. Eight new sequences were obtained in this study. Phylogenetic relationships of *M. andina* gen. n., comb. n. with other Heteroderidae are given in Figure 5. The gene sequence of *M. andina* gen. n., comb. n. formed a clade (PP = 99%) with that of cyst nematode, *Vittatidera zeaphila* and differed from it in 79 bp (19.5%).

The partial *cytb* mtDNA gene. Blastn results showed that the *cytb* nucleotide gene sequence of *M. andina* gen. n., comb. n. (248 bp) was similar with those of *Heterodera glycines* (HM640930) in 82.1% (coverage 96%) and with *Globodera capensis* (MN096070) in 81.5% (95%).

Maximum likelihood testing. The results of ML testing of tree topologies and alternative phylogenetic hypotheses: *i*) a sister relationship of *Mesodolichodera* gen. n. and the subfamily Punctoderinae; *ii*) a sister relationship of *Mesodolichodera* gen. n. and cyst nematodes (subfamilies Heteroderinae + Punctoderinae); and *iii*) *Mesodolichodera* gen. n. is a representative of the subfamily Ataloderinae are given in Table 2. All ML tests with the ITS rRNA gene sequences rejected all tested alternative topologies, whereas these tests with the D2-D3 of 28S rRNA and *COI* gene sequences did not exclude alternative topologies.

Wouts (1985) already stated that since no cyst was formed and the female cuticle from cystoid nematode from Peru was partially annulated; it did not seem completely identical to *Dolichodera*; it appeared more primitive and may represent an independent genus. The phylogenetic analysis of rRNA and *COI* genes amplified from females showed that this species represents an independent evolutionary lineage closely related with cyst-forming nematodes. Based on molecular results and unique morphological characters we suggested that this nematode should belong to a new genus and a new subfamily, which are erected below.

Mesodolichodera gen. n.

Diagnosis. Family Heteroderidae; Subfamily Mesodolichoderinae subfam. n. **Mature female.** Body round to oval, smooth and rounded posteriorly, with small neck protruding anteriorly, white, becoming yellowish in some specimens. No cyst formed. Eggs retained in body. Egg sac not observed. Head offset, with four annuli and a labial disc. Stylet strong, with heavy, large knobs sloping posteriorly. Cuticular surface in posterior part of body, and especially in the vulval-anal area, consists of a lace-like pattern overlaying random punctation. Anus and vulva are terminal, well separated in distance of 44-91 µm from each other. Vulval slit short (6-8 µm), surrounded by a small smooth area with 4-5 annuli. Inner vulval lips faint, outer vulval lips absent. **Male.** Body not twisted. Head region faintly annulated, lateral field with four incisures. Phasmids not seen. Tail short, rounded. **Second-stage juvenile.** Body short, 362-437 µm in a length. Pharyngeal glands not restricted to ventral side of body. Lateral field with four incisures, forming three bands of equal width, outer band areolated. Phasmids occurs as a small pit in the middle longitudinal band, with lens-like structure. Tail short, conically tapering to a rounded terminus.

Type and only species:

Mesodolichodera andina (Golden, Franco, Jatala & Astogaza, 1983) gen. n., comb. n.

= *Thecavermiculatus andinus* Golden, Franco, Jatala & Astogaza, 1983.

= *Dolichodera andinus* (Golden, Franco, Jatala & Astogaza, 1983) Wouts, 1985.

= *Atalodera andina* (Golden, Franco, Jatala & Astogaza, 1983) de Souza & Huang, 1994.

Differential diagnosis and relationships. Morphological characters, such as absence of cyst stage, long vulva-anus distance and short vulval slit place *Mesodolichodera* gen. n. in a new subfamily *Mesodolichoderinae* subfam. n. *Mesodolichodera andina* gen. n., comb. n. has faint inner vulval lips and lacks outer vulval lips in females. It is also distinct by the fine lace-like cuticular pattern in the mid-body region of females. Second-stage juvenile has the lateral field which continues to the posterior terminus as three bands with each minute phasmid opening located in the centre of the middle band (Othman *et al.*, 1986). Analysis of the rRNA and *COI* gene sequences showed that *Mesodolichodera* gen. n. shares a unique clade with two cyst nematode genera: *Betulodera* Sturhan, 2002 and *Vittatidera* Bernard, Handoo, Powers, Donald & Heinz, 2010.

Mesodolichoderinae subfam. n.

Diagnosis. Family Heteroderidae. No cyst formed. Female body contains eggs and egg sac not observed. Anus and vulva are terminal and well separated from each other. Vulval slit short.

Differential diagnosis. New subfamily differs from related the subfamily Punctoderinae Krall & Krall, 1978 by absence of cyst stage from the subfamily Ataloderinae Wouts, 1973 by long vulva-anus distance and short vulval slit.

The cystoid nematode, *Ekphymatodera thomasoni* Baldwin, Bernard & Mundo-Ocampo, 1989, representative of the subfamily Ataloderinae, strongly clustered with cyst-forming nematodes, but not with the genera *Atalodera* and *Rhizonemella* and it makes this subfamily paraphyletic. It has been proposed that the subfamily Ataloderinae might require a revision with the erection of a new subfamily for *E. thomasoni* (Subbotin *et al.*, 2017). In our present study, the close relationships of another cystoid nematode *M. andina* gen. n., comb. n, with cyst-forming nematodes were also revealed. Morphological and phylogenetic analysis of several genes justified a placement of this taxon in new genus and subfamily. Thus, the results showed that at least two cyst-forming nematode genera clustered

within cyst nematode genera and the previous statement that cyst formation appeared only once during the evolution of Heteroderidae (Subbotin & Skantar, 2018) requires additional verification and testing.

Our results showed a close relationship of *M. andina* gen. n., comb. n. inhabiting the Andes mountains with the two cyst-forming nematode genera: *Betulodera* and *Vittatidera* from North America. It has been proposed that North America was the predominant centre of origin for the cystoid nematodes and secondary centre of speciation for this group might be in the mountains of northern Vietnam and southern China of the Indo-Burma Biodiversity hotspot (Subbotin *et al.*, 2017). Present results indicate that the Andes might be considered as another secondary centre of speciation for cystoid nematodes and, thus, phylogeography of cyst and cystoid nematodes are more complex than has been previously proposed.

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S.A. Suubbotin and J. Franco. Молекулярная характеристика цистоидной нематоды *Mesodolichodera andina* (Golden, Franco, Jatala & Astogaza, 1983) gen. n., comb. n. (Tylenchida: Heteroderidae), паразитирующий на картофеле в Андах.

Резюме. Молекулярная характеристика цистоидной нематоды *Mesodolichodera andina* gen. n., comb. n., паразитирующей на корнях картофеля, собранного на картофельном поле в округе Покси, провинция Арекипа (Перу), дается на основе последовательностей генов D2-D3 28S рРНК, ITS рРНК, *COI* и *cytb*. Эта нематода ранее была известна как *Thecavermiculatus andinus*, *Dolichodera andina* или *Atalodera andina*. *Mesodolichodera andina* gen. n., comb. n. обнаружена в смеси с *Globodera rostochiensis*. Филогенетический анализ генов рРНК и *COI Mesodolichodera andina* gen. n., comb. n. показал, что эта нематода представляет собой независимую эволюционную линию, тесно связанную с цистообразующими нематодами родов *Betulodera* и *Vittatidera*. На основании молекулярных результатов и анализа уникальных морфологических признаков было высказано предположение о принадлежности этой нематоды к новому роду и новому подсемейству семейства Heteroderidae. *Atalodera* spp. и *Rhizonemella* spp. собранные из разных мест в Орегоне и Калифорнии, США, также были молекулярно охарактеризованы несколькими маркерами в этом исследовании. Филогенетические отношения внутри семейства Heteroderidae были реконструированы с использованием последовательностей генов рРНК и *COI*.