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Molecular characterisation and phylogenetic relationship of *Verutus volvingentis* Esser, 1981 with other cystoid nematodes of the family Heteroderidae (Nematoda: Tylenchida), and some morphological details of its immature life stages

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Summary. New phylogenies of cystoid heteroderid nematodes, a group characterised by females that do not turn into hard-walled cysts, were obtained using the D2-D3 of 28S rRNA, ITS rRNA, partial 18S rRNA and partial *COI* mtDNA gene sequences. The cystoid nematodes studied included a Florida population of *Verutus volvingentis* from the type locality, an unidentified *Verutus* sp. from the USA, *Cryphodera* sp. D from Thailand, *Zelandodera* sp. from New Zealand and an unknown and putatively new genus of the family Verutinae from Borneo, Malaysia. The results of these analyses indicated that the subfamily Verutinae, represented by species of the genus *Verutus*, is phylogenetically related to the family Heteroderidae rather than Rotylenchulidae. *Verutus* species sequences clustered in well supported clades separated from those of other cystoid nematodes. Our study confirmed the unique status of a cystoid nematode from Borneo attributed by D. Sturhan (2018) to an unknown genus in Heteroderidae. In the phylogenetic trees obtained using the studied gene sequences, the genus *Zelandodera* formed a distinct lineage within Meloidoderinae, confirming the validity of this genus that has not previously been accepted by some authors. Light microscopic studies of *V. volvingentis* showed the presence of transparent first-stage juvenile moulted cuticle inside the eggs indicating that second-stage juveniles hatch from the eggs. Phasmids in the second-stage juvenile tail were pore-like and connected to a fine duct.

Key words: Borneo, *COI* mtDNA, *Cryphodera*, D2-D3, Florida, ITS rRNA, *Meloidodera*, New Zealand, 18S rRNA, phasmids, post-embryonic development, systematics, Thailand, Verutinae, *Zelandodera*.

Verutus volvingentis Esser, 1981, was first found in 1969, during regulatory analyses, conducted by the Florida Department of Agriculture and Consumer Services. The analysed soil and root samples infested by the nematode were from buttonweed, *Diodia virginiana* L. collected in a field near Apopka. The species was, however, formally described twelve years after its discovery (Esser, 1981). The semi-endoparasitic habits of *V. volvingentis* were elucidated in the original description that includes detailed anatomical features of all the life stages. Juvenile females hatching from the eggs have a robust stylet. They penetrate the host roots with the anterior portion of the body leaving the posterior portion protruding from the root surface (Cohn *et al.*, 1984). They develop into sausage or reniform shaped females having an uncommonly large vulva in a subequatorial position. Deposited eggs are viscose and adhere to the female's body cuticle. The eggs are not enclosed inside a gelatinous matrix and nor are they retained inside the female body, which does not become a cyst. These morphological and biological characteristics are shared in part by species in both families Heteroderidae and Rotylenchulidae. Esser (1981) considered these features more related to those of the family Heteroderidae than Rotylenchulidae and proposed

the inclusion of *V. volvingentis* in a new subfamily, Verutinae, inside the family Heteroderidae. This classification was accepted by many taxonomists including Otham & Baldwin (1985), Luc *et al.* (1988), Baldwin *et al.* (1989) and Sturhan (2018). Other taxonomists such as Siddiqi (1986, 2000) and Decraemer & Hunt (2013) did not share this view and included the subfamily Verutinae in the family Rotylenchulidae.

Verutus volvingentis is a well described species, but some aspects of its description require clarification. Esser (1981) stated that, after the post-embryogenic development, the first-stage juvenile (J1) hatches from the egg without moulting. No moulted cuticle was observable inside the egg with the compound microscope he used. He also concluded the description by stating that: "The principal character of the first stage larva of the new genus (*Verutus*) is the absence of a detectable phasmid". Othman & Baldwin (1985) and Baldwin *et al.* (1989) clarified Esser's controversial statements and stated that phasmid openings in *V. volvingentis* are observable with SEM and reduced internal structures can be seen when examined with TEM. The SEM illustrations published by Otham & Baldwin (1985) show a superficial view of the phasmids that appear as pit-like structures. Phasmids with similar shape were reported by Baldwin *et al.* (1989) in the description of *V. californicus*, a new species detected in California, but not included in our study for lack of specimens. In a study on the structures of phasmids and lateral field of heteroderid second-stage juvenile (J2) to be used for diagnostic purpose, Sturhan (2018) observed pore-like phasmids in *V. volvingentis* paratypes. He also attempted to verify phylogenetic relationships based on the shapes of these morphological structures in J2 of the species of subfamilies in Heteroderidae.

So far, the molecular characters and the phylogenetic relationship based on DNA sequences of *V. volvingentis* with members of the Heteroderidae and Rotylenchulidae families have not been elucidated. The results of preliminary phylogenetic studies reported by Baldwin *et al.* (1989), as well by Subbotin *et al.* (2017) using DNA sequences of an undescribed *Verutus* from Germany, suggested that the species of *Verutus* clustered in a basal position within all other Heteroderidae. However, these findings should be confirmed with more representatives of this subfamily. New molecular data are also needed on other cystoid nematodes, such as species of *Cryphodera* and *Zelandodera* to clarify their taxonomic status and relationships. As far as we know, no postembryonic developmental studies have been conducted to disprove Esser's statement that J1 hatches from the egg in *V. volvingentis*, even if J2 is considered the motile life stage that hatches from the eggs in *Verutus* sp., including *V. volvingentis* (Otham & Baldwin, 1985; Luc *et al.*, 1988; Baldwin *et al.*, 1989). Clarification of these morphological aspects of *V. volvingentis* is needed to confirm or disprove Esser's statements.

Table 1. Species of cystoid nematodes of the family Heteroderidae used in this study.

Species	Locality	Host	Sample code	GenBank accession number				Source or reference
				18S rRNA	D2-D3 of 28S rRNA	ITS rRNA	*COI*	
Verutus volvingentis	USA, Florida, Oxford	*Diodia virginiana*	CD2523	MK033151	MK033159	MK033166	MK033153	R.N. Inserra
Verutus sp. B	USA	Unknown plant	CD2721	MK033150	MK033160	MK033165	MK033154	S.A. Subbotin
Cryphodera sp. D	Thailand, Surat Thani, Khlong Sok, Our Jungle House resort. GPS: 8°54'31''N, 98°32'1''E	Unknown plant	CD2750	MK033149	MK033162	MK033163	MK033156	S.A. Subbotin, C. Borkent
Zelandodera sp.	New Zealand	Unknown plant	CD2535	–	MK033161	–	MK033157	D. Sturhan
Cystoid nematode	Malaysia, Borneo, Sarawak	Unknown plant	CD2526 CD2532	MK033152	MK033158	MK033164	MK033155	D. Sturhan

The main objectives of the present study were: *i*) to provide new information, using light microscopy, about the postembryonic development of *V. volvingentis* and the shape of the juvenile phasmid; *ii*) to provide molecular characterisation of *V. volvingentis* using D2-D3 of 28S rRNA, ITS rRNA, partial 18S rRNA and partial *COI* mtDNA gene sequences; *iii*) to provide molecular characterisation of other cystoid nematode species including another unidentified *Verutus* species from an unknown locality in the USA, unidentified species of *Cryphodera* from Thailand and *Zelandodera* from New Zealand, and a representative of a new cystoid nematode genus from Malaysia using these four gene fragments; and *iv*) to analyse phylogenetic relationships of these species with other representatives of the family Rotylenchulidae and Heteroderidae using these rRNA and *COI* gene fragments.

MATERIAL AND METHODS

Nematode samples. The nematode populations used in this study included: specimens of *V. volvingentis* obtained from buttonweed plants growing in a peat mine in Central Florida, in an area close to the type locality; an unidentified *Verutus* sp. from a soil sample collected in the USA from an unknown plant shipped to the Plant Pest Diagnostic Centre, California Department of Food and Agriculture; and another nematode belonging to a putatively new genus of the subfamily Verutinae provided by Dr D. Sturhan from samples collected in Borneo, Malaysia. Two representatives of the subfamily Meloidoderinae were also included in this study (Table 1). Nematode-infested buttonweed plants from the peat operation were kept in the same original peat in a glasshouse to provide nematode specimens and infested roots for biological and behavioural observations.

Light microscopic study. Feeder root segments removed from buttonweed plants were placed in Petri dishes containing water and examined at light microscopy (LM) using a stereo microscope to obtain eggs surrounding the body of swollen females. Embryonated eggs (112) were picked singly, placed on water agar, sealed with a cover slip and examined using a compound microscope with an oil immersion objective (Esser, 1986) to observe J1 moulting inside the eggs. Nematode J2 hatched from these eggs or extracted from soil using the centrifugal-flotation method (Jenkins, 1964) were immobilised by gently heating and then mounted on water agar and observed as described above to examine the phasmids. The morphometrics of these specimens fit those of the original description and are not reported. Measurements of an undescribed *Verutus* population were taken with an ocular micrometer. Some J2 were frozen (–20°C) for molecular study. Light micrographs of this *Verutus* and other cystoid nematodes were taken 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. The morphometric values of the undescribed *Verutus* J2 were compared with those of *V. californicus* and *V. volvingentis*. Additional micrographs of eggs and phasmids in J2 were taken with an equivalent Zeiss compound microscope with similar Nomarski equipment.

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) and Subbotin *et al.* (2017). 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) 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') for amplification of the partial 18S 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') primers (Subbotin, 2015) for amplification of the partial *COI* gene.

PCR products of three nematode samples were sequenced directly, whereas PCR products of two *Verutus* species were cloned. The PCR products were purified using QIAquick (Qiagen) Gel extraction kits and cloned using pGEM-T Vector System II kit (Promega). One clone from each

sample was sequenced. Sequencing was conducted at Quintara Biosciences, CA, USA. The newly obtained sequences were submitted to the GenBank database under accession numbers: MK033149-MK033166 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, 18S rRNA and the *COI* mtDNA) were aligned using Clustal_X (Thompson *et al.*, 1997) with their corresponding published gene sequences (Subbotin *et al.*, 2001, 2006, 2017; Ferris *et al.*, 2004; Nguyen *et al.*, 2011; Vovlas *et al.*, 2013; Zhuo *et al.*, 2014a, b; van den Berg *et al.*, 2016; and others). Outgroup taxa, representatives of the genera *Scutellonema*, *Rotylenchus* and *Hoplolaimus*, for each dataset were chosen based on previously published data (Subbotin *et al.*, 2006, 2017). Clustal_X was run with default parameters. Two ITS rRNA alignments were used for the analysis: *i*) full length alignment, and *ii*) culled alignment, an alignment without poorly aligned positions and divergent regions that were removed with Gblocks software (Talavera & Castresana, 2007). 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 values (BS) for ML tree were calculated by 'faststep' search from 100 replicates. 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. For testing of alternative topologies in ML we used the Shimodaira-Hasegawa (SH) test as implemented in PAUP*.

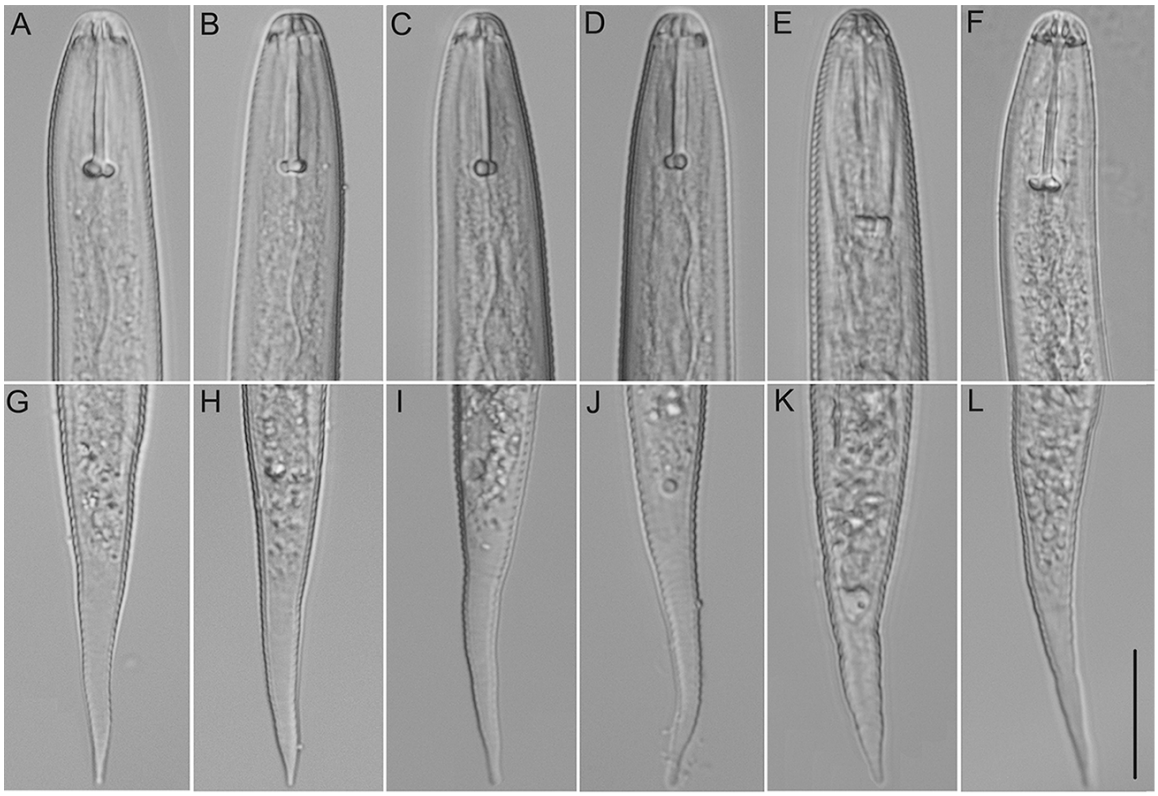

Fig. 1. Photomicrographs of anterior and posterior regions of second-stage juveniles. A, B, G, H: *Verutus volvingentis* (CD2523); C, D, I, J: *Verutus* sp. B (CD2721); E, K: *Zelandodera* sp. B (CD2535); F, L: *Cryphodera* sp. D (CD2750). Scale bar = 20 µm.

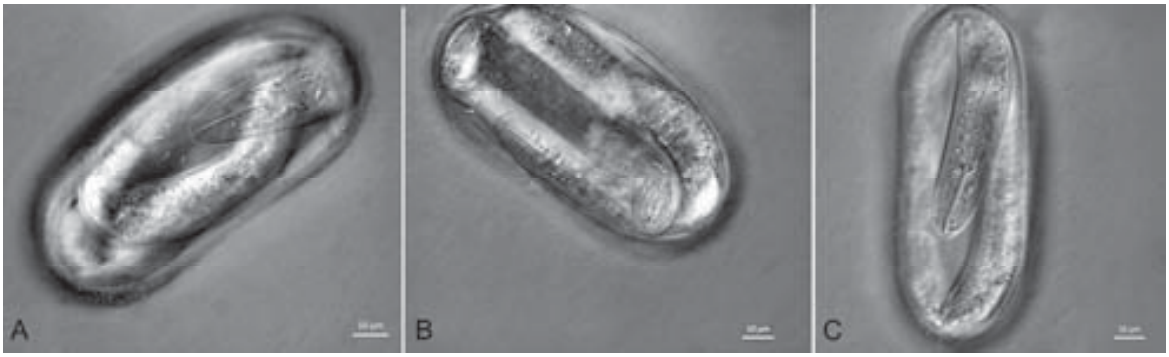

Fig. 2. Photomicrographs of embryonated eggs of *Verutus volvingentis*. A, B: Coiled second-stage juveniles (J2) showing a transparent moulted cuticle (arrowed) of the first-stage juvenile surrounding the anterior portion of their body, which lacks the cephalic framework; C: Coiled J2 showing the anterior body portion with developed framework.

RESULTS

Light microscopic study. Light microscopic photographs of anterior and posteriors regions of J2 for *Verutus volvingentis* and five other unidentified species used for the molecular and phylogenetic studies are presented in Figure 1.

Selective measurements of J2 of an undescribed *Verutus* sp. B (USA) included: (n = 5): L= 526.5 ± 37.4 (462.5-552.5) µm; W = 20.8 ± 0.6 (20.0-21.3) µm; a = 25.2 ± 1.4 (23.1-26.8); b = 3.3 ± 0.2 (3.1-3.5); c = 8.6 ± 0.7 (7.7-9.6); c' = 1.7 ± 0.1 (1.6-1.9); stylet = 24.1 ± 0.8 (23.1-25) µm; pharynx = 161 ± 17.6 (137.5-180.0) µm; anterior end to median bulb = 45.7 ± 2.5 (42.9-49.1) µm; tail = 61.5 ± 3.8 (56.3-66.3) µm; hyaline part of tail = 35.3 ± 2.6 (31.3-37.5) µm.

These measurements differed from those of *V. californicus* J2, which have shorter body (526.5 (462.5-552.5) *vs* 622.2 (590-670) µm) and tail (61.5 (56.3-66.30) *vs* 81.7 (75-91) µm). They are, however, closer to those of *V. volvingentis*, which have shorter hyaline portion of the tail [25.4 (23.5-31.3 µm)] than that of the undescribed *Verutus* sp. B [35.3 (31.3-37.5 µm)]. Because of the overlapping of J2 measurements in these two species, supplemental molecular analyses are required for their separation. The analyses of the DNA sequences of these two *Verutus* show that they represent two distinct species.

Observations of the embryonated eggs of *V. volvingentis* showed fully developed J2. Moulted J1 cuticles were difficult to detect. The juveniles completed their development inside the egg without showing the noticeable cuticular cap surrounding the cephalic portion of the moulted J1 as has been reported for other Tylenchida (Inserra *et al.*, 1983, 1993). A faint cuticular cap surrounding the cephalic region was, however, observed in five percent of the juveniles having a developed stylet and before the formation of their cephalic framework. This faint cuticular cap may be considered as a vestigial moulted cuticle of the first-stage juvenile, confirming the views of Luc *et al.* (1988) and Baldwin *et al.* (1989) that J2 of *V. volvingentis*, like those of other *Verutus* spp, hatch from the eggs (Fig. 2). The examination of J2 tail allowed the detection of the phasmids that were located posteriorly to anus at about 30% of the tail length. Phasmids were minute without any lens-like ampulla. A fine duct connected to the phasmids was detected by focusing deeply below the phasmid aperture (Fig. 3).

Molecular characterisation and phylogenetic relationships. The D2-D3 of 28S rRNA gene. The D2-D3 alignment was 686 bp long and consisted of 44 sequences including two sequences of outgroup species. Phylogenetic relationships within Heteroderidae and Rotylenchulidae are given in Figure 4. The phylogenetic tree contained five major clades, three of them were highly supported in BI (PP = 100%) and highly or moderate supported in ML (BS > 76%): *i*) Heteroderinae and Punctoderinae, *ii*) Ataloderinae, *iii*) Rotylenchulidae; one moderately supported in BI (PP = 89%) and weakly supported in ML (BS < 50%) clade: *iv*) Verutinae containing three *Verutus* species and a cystoid nematode from Malaysia and a weakly supported clade (PP and BS < 50%): *v*) Meloidoderinae containing *Meloidodera*, *Cryphodera* and *Zelandodera* representatives. The clade I with cyst nematodes clustered with the clade II with a high support in BI (PP = 100%), whereas the relationships between IV and V clades remain unresolved. *Verutus volvingentis* formed a highly supported clade (PP = 100%; BS = 99%) with other *Verutus* species. The sequence of *V. volvingentis*

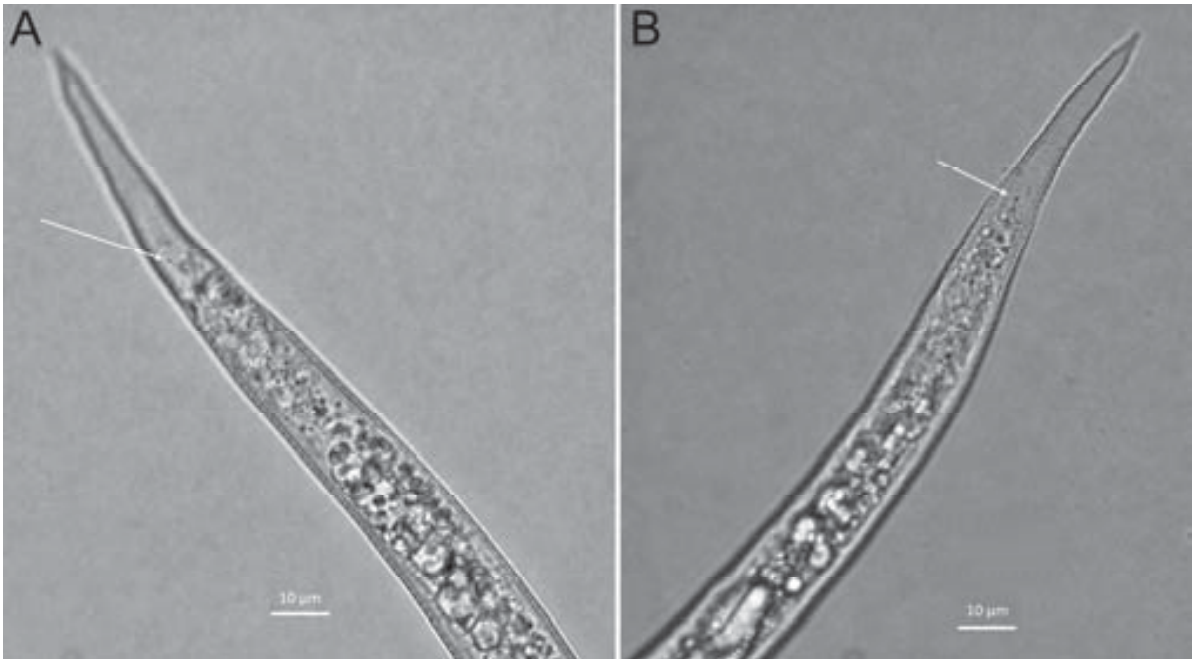

Fig. 3. Photomicrographs of *Verutus volvingentis* second-stage juvenile. A, B: Posterior body portion showing pore-like phasmids connected to a fine duct (arrowed).

differed from those of *Verutus* sp. A (Germany) in 7.9% (52 bp) and from *Verutus* sp. B (USA) in 7.3% (47 bp). The sequences of *Verutus* sp. A and *Verutus* sp. B differed in 3.2% (21 bp).

The ITS of the rRNA gene. The ITS alignment was 1202 bp long and consisted of 25 sequences including two sequences of outgroup species. After removing ambiguously aligned positions and divergent regions using Gblocks, the ITS alignment became 372 bp in a length. Phylogenetic relationships within Heteroderidae and Rotylenchulidae as inferred from both alignments are given in Figure 5A and B. The trees contained four (Fig. 5A) or five (Fig. 5B) major clades and at least three of them were highly supported. The trees included the following groups: *i*) Ataloderinae, *ii*) Meloidoderinae and a cystoid nematode from Malaysia, *iii*) Verutinae and *iv*) Rotylenchulidae. *Bilobodera flexa* formed a highly supported clade with two *Verutus* species in the BI tree inferred from the full length ITS alignment, whereas it clustered with Ataloderinae in the tree inferred from the culled alignment. The sequence of *V. volvingentis* differed from that of *Verutus* sp. B (USA) in 22.2% (195 bp) for the full length of ITS rRNA gene sequence. The ITS1 rRNA gene sequences of *Cryphodera* sp. D from Vietnam and Thailand were different in 3.5% (21 bp).

The 18S rRNA gene. The 18S alignment was 802 bp long and consisted of 15 sequences including two outgroup species. Phylogenetic relationships within Heteroderidae were not well resolved and are presented in Figure 6.

***COI* mtDNA.** The alignment was 478 bp long and contained 29 sequences, including two *Rotylenchus* sequences as outgroups. Phylogenetic relationships are given in Figure 7. The BI phylogenetic tree contained several well supported major clades: *i*) Ataloderinae and a cystoid nematode from Malaysia, *ii*) Heteroderinae and Punctoderinae, *iii*) Meloidoderinae, *iv*) *Verutus* spp, and *v*) Rotylenchulidae. The sequence of *V. volvingentis* differed from *Verutus* sp. B (USA) in 9.5% (42 bp). Sequences of *Cryphodera* sp. D from Vietnam and Thailand were different in 0.2% (1 bp).

Maximum likelihood testing. Results of the Shimodaira-Hasegawa test of tree topologies and alternative phylogenetic hypotheses of Heteroderidae are given in Table 2. The SH testing of an alternative topology with three gene fragments (D2-D3 of 28S rRNA, ITS rRNA and *COI* mtDNA) does not reject the non-monophyly for *Meloidodera*. The monophyly of Verutinae including a cystoid nematode from Malaysia was also supported, except when the full alignment of ITS rRNA gene was tested (Table 2).

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

Hypothesis	D2 and D3 of 28S rRNA			ITS rRNA (full alignment)			ITS rRNA (culled alignment)			COI mtDNA		
	-ln L	Δ ln L	P	-ln L	Δ ln L	P	-ln L	Δ ln L	P	-ln L	Δ ln L	P
ML tree	8392.98	best	–	16935.60	best	–	3015.49	best	–	5343.98	best	–
Verutinae is monophyletic	8392.98	0.0	1.000	16965.77	30.172	0.000*	3021.06	5.566	0.311	5347.42	3.448	0.438
Meloidodera is monophyletic	8414.93	21.943	0.068	16945.65	10.050	0.159	3023.63	8.135	0.207	5352.02	8.040	0.257

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

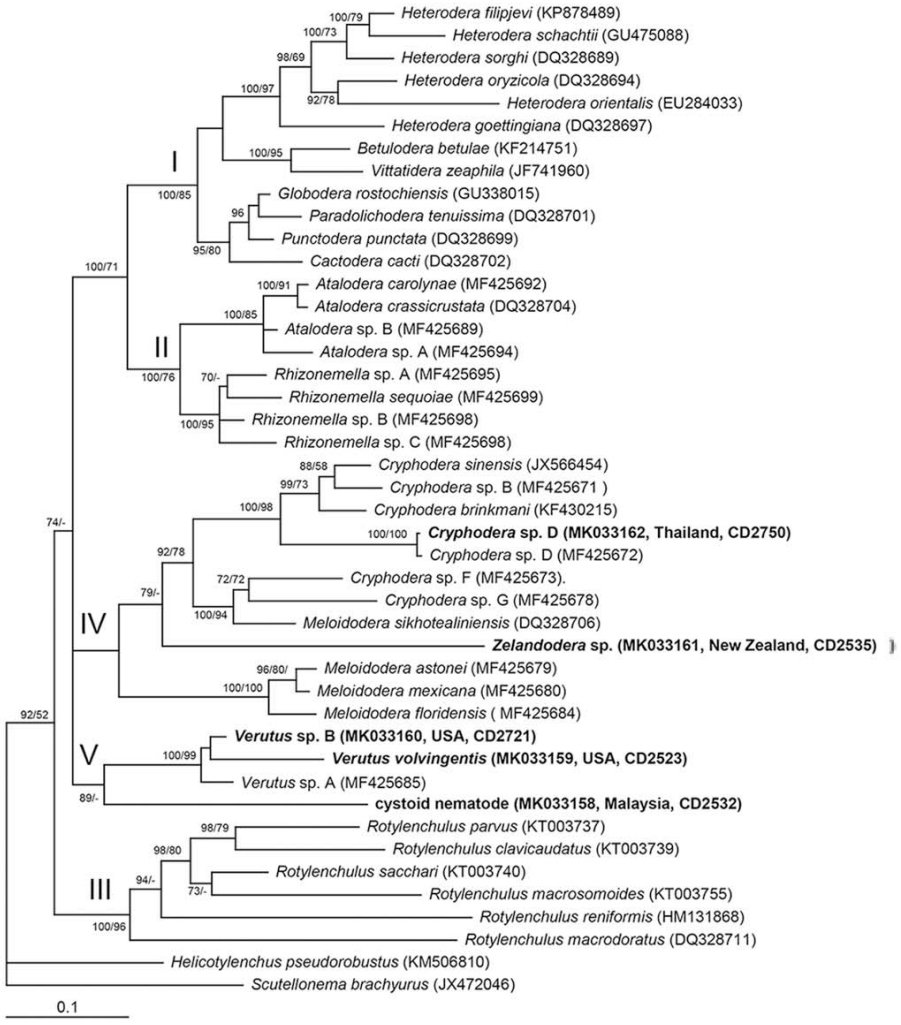

Fig. 4. Phylogenetic relationships within Heteroderidae and Rotylenchulidae: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the D2-D3 of 28S rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities equal to, or more than, 70% and bootstrap values are given for appropriate clades. Original sequences are indicated by bold font.

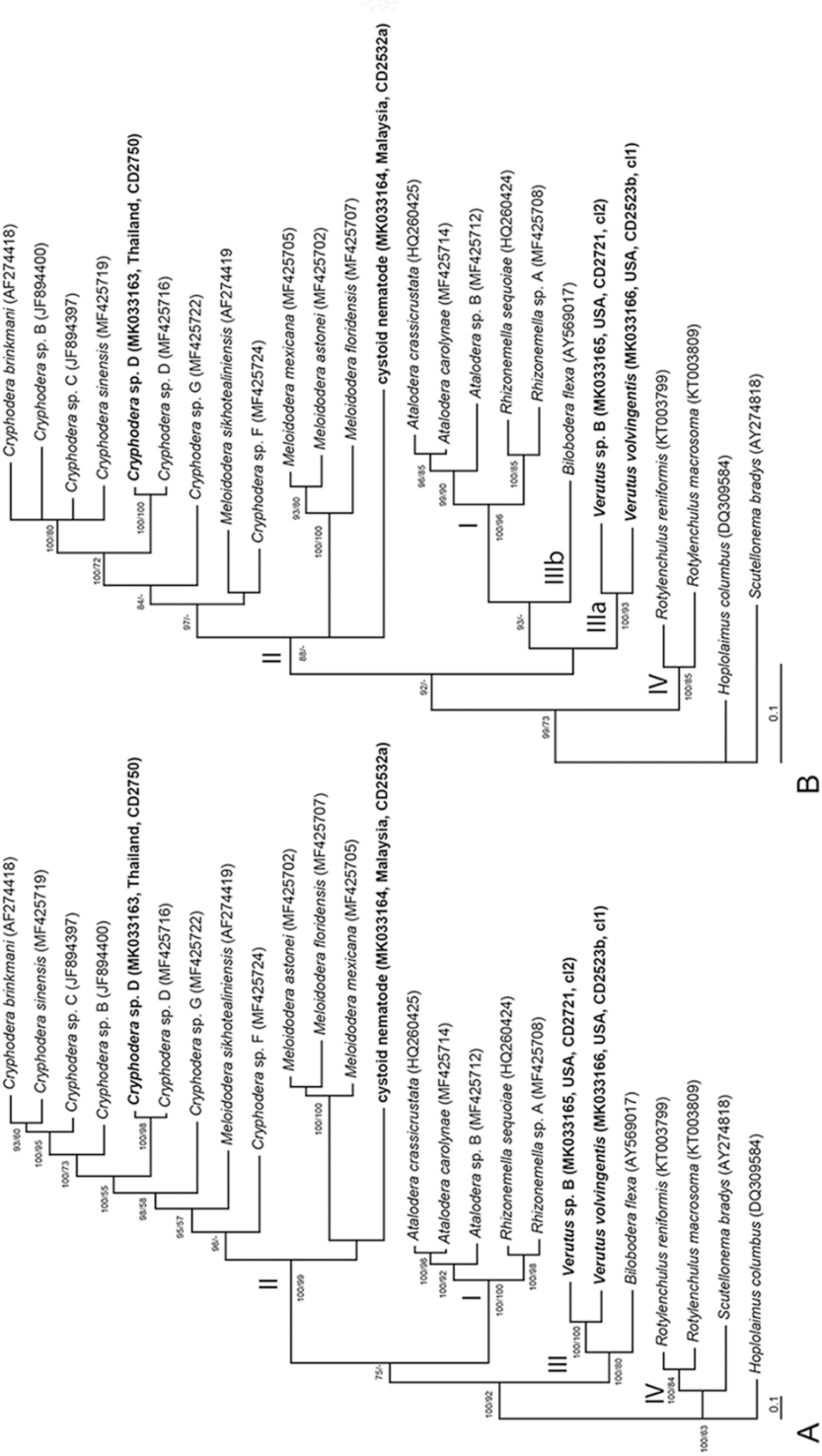

Fig. 5. Phylogenetic relationships within Heteroderidae and Rotylenchulidae: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the ITS rRNA gene sequence alignment under the GTR + I + G model. A: Full length alignment; B: Culled alignment with exclusions of poorly aligned positions and divergent regions. Posterior probabilities equal to, or more than, 70% and bootstrap values are given for appropriate clades. Original sequences are indicated by bold font

DISCUSSION

The results of the phylogenetic analysis conducted in this study using four gene markers indicated that the cystoid nematode of the subfamily Verutinae is phylogenetically related to other representatives of the family Heteroderidae rather than to Rotylenchulidae. It confirms the finding of a recent study by Subbotin *et al.* (2017) but now with larger number of representatives of Verutinae.

Subbotin *et al.* (2017) and our present study showed that the genus *Meloidodera* was non-monophyletic in the trees reconstructed from both rRNA and *COI* gene sequence datasets and that their representatives were distributed within two clades. The first clade in our trees includes *M. sikhotealiniensis* from Asia and Europe and *Cryphodera* spp. found in Asia; the second clade contains *M. astonei*, *M. floridensis* and *M. mexicana*, which are all species from North America. Subbotin *et al.* (2017) proposed that a taxonomic revision of *Meloidodera* was required to define taxa on the basis of phylogenetic groups inferred from morphology and molecular datasets. However, the present ML testing revealed that the trees with an enforced monophyly of the genus *Meloidodera* are not significantly worse that the best ML tree; thus, a monophyly of the genus could be still considered from molecular datasets.

Our light microscopy study clarified the controversial statements in the original descriptions of *V. volvingentis*. Our observations provided evidence that the postembryonic development of *V. volvingetis* includes the moulting of J1 to J2 in the eggs. However, moulted cuticles of J1 were transparent and undetectable without the use of a compound microscope equipped with Nomarski differential interference contrast. This microscopic equipment also allowed the detection of the phasmids in the J2. These structures were pore-like and connected to a duct. The shape of the phasmids is considered a useful diagnostic character for the separation of *Verutus* J2 from those of other cystoid nematodes (Sturhan, 2018).

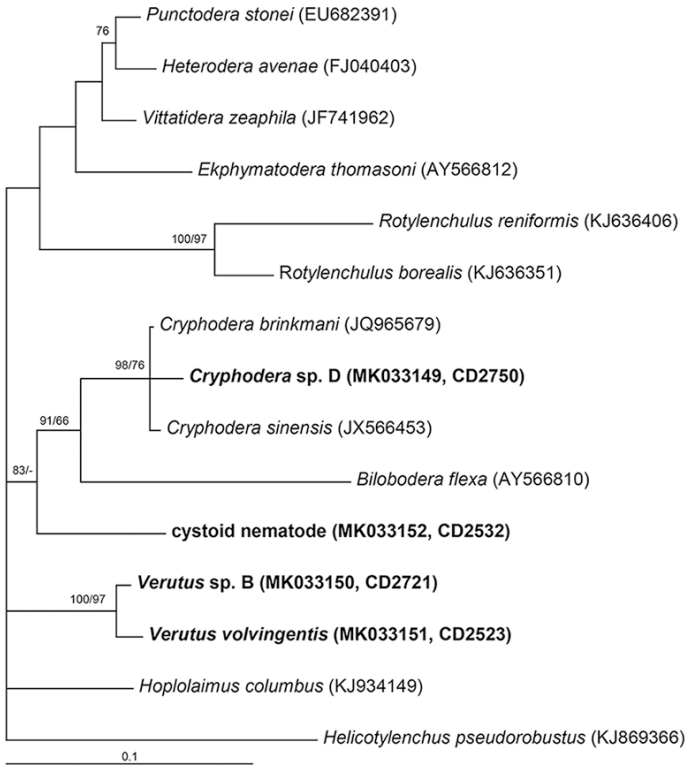

Fig. 6. Phylogenetic relationships within Heteroderidae: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the partial 18S rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities equal to, or more than, 70% and bootstrap values are given for appropriate clades. Original sequences are indicated by bold font.

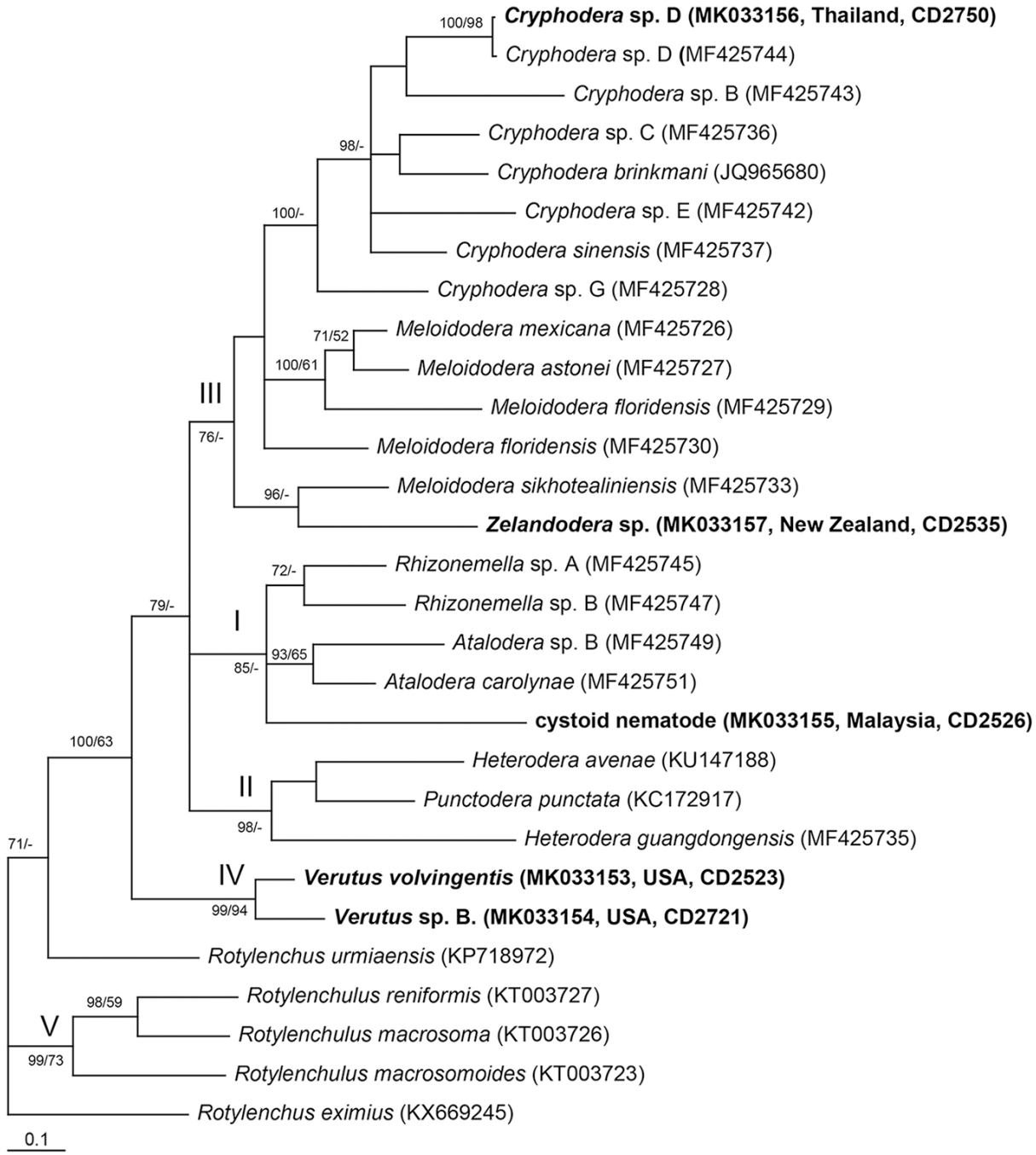

Fig. 7. Phylogenetic relationships within Heteroderidae and Rotylenchulidae: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the *COI* mtDNA gene sequence alignment under the GTR + I + G model. Posterior probabilities equal to or more than, 70% are and bootstrap values given for appropriate clades. Original sequences are indicated by bold font.

Using molecular techniques in this study, we characterised a cystoid nematode from Borneo, Malaysia provided by D. Sturhan, who noticed that this nematode could not be attributed to any of the known genera in Heteroderidae (Sturhan, 2018). The continuous lip region and the moderately developed cephalic framework suggested putative placement of this genus in the subfamily Verutinae, although other morphological characters were distinctly different from those of all species known in Verutinae. Our molecular analysis confirmed the unique taxonomic status of this nematode and placed it in Verutinae in the partial 28S rRNA gene tree, or in Meloidoderinae in the ITS rRNA gene tree or in Ataloderinae in the *COI* gene tree, thus making its phylogenetic position within Heteroderidae uncertain. However, the SH test with all studied gene fragments does not exclude its grouping with Verutinae and, thus, Sturhan's hypothesis on placing this nematode in the subfamily Verutinae cannot be rejected from the present molecular datasets.

A cystoid nematode from New Zealand was preliminary morphologically identified by D. Sturhan as *Cryphodera* sp., but it was named here as a representative of *Zelandodera*, a genus closely related to *Cryphodera*. Wouts (1973) established the genus *Zelandodera* and separated it from *Cryphodera* for having females with anus located in the dorsal curvature of their body and less pronounced vulval lips; J2 with greater number of lip annuli (4-5 *vs* 3), and males with more incisures in the lateral field (4 *vs* 3). However, Luc *et al*. (1978) considered these differences insufficient for the establishment of a new genus and synonymised *Zelandodera* with *Cryphodera*. This synonymy was supported by Siddiqi (1996, 2000), but was not accepted by Wouts (1985) and Krall (1989). Sturhan (2018) did not recognise the genus *Zelandodera* as a separate taxon, but distinguished the *Zelandodera* group within the genus *Cryphodera*. Our analysis showed that *Zelandodera* may represent a distinct lineage within Meloidoderinae, although more species of this family from New Zealand, Asia and North America should be included in future study.

As a concluding remark, we would like to add that in this study we provide additional evidence of the wide geographical distribution of the species of the genus *Verutus*, which evolved in wet environments in different continents. Our new molecular and phylogenetic evidence supplements the study conducted by Sturhan (2018) concerning the taxonomic status of the species of cystoid nematodes described in his diagnostic compendium.

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S.A. Subbotin, S. Vau and R.N. Inserra. Молекулярная характеристика и филогенетические взаимоотношения *Verutus volvingentis* Esser, 1981 с другими цистоидными нематодами семейства Heteroderidae (Nematoda: Tylenchida) и некоторые морфологические детали личиночных стадий этой нематоды.

Резюме. Новые филогенетические схемы для цистоидных гетеродеридных нематод – группы, характеризующейся наличием самок, которые не превращаются в жесткостенные цисты – были получены с использованием анализа генов D2-D3 28S рРНК, ITS рРНК, частичной 18S рРНК и *COI* митохондриальной ДНК. Изученные цистоидные нематоды включали флоридскую популяцию *Verutus volvingentis* из типового местообитания, не определённые до вида *Verutus* sp. из США, *Cryphodera* sp. D из Таиланда, *Zelandodera* sp. из Новой Зеландии и представителя неизвестного и предположительно нового рода семейства Verutinae из Борнео, Малайзия. Результаты этих анализов показали, что подсемейство Verutinae, представленное видами рода *Verutus*, филогенетически связано с семейством Heteroderidae, а не с Rotylenchulidae. Виды рода *Verutus* были сгруппированы в отдельную от других цистоидных нематод хорошо поддерживаемую кладу. Наше исследование подтвердило уникальный статус цистоидной нематоды из Борнео, отнесенной Д. Штурханом (2018) к неизвестному роду семейства Heteroderidae. На филогенетических деревьях, полученных в результате анализа изученных последовательностей генов, род *Zelandodera* сформировал отдельную ветвь в пределах Meloidoderinae, подтверждая обоснованность выделения этого рода, который, однако, ранее не был принят некоторыми авторами. Светомикроскопические исследования *V. volvingentis* показали наличие прозрачной кутикулы личинки первой стадии внутри яиц, что указывает на то, что из яиц вылупляются личинки второй стадии. Фазмиды в хвосте личинки второй стадии поровидные и соединены с тонким каналом.