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Trefusiidae Are a Subtaxon of Marine Enoplida (Nematoda): Evidence from Primary Structure of Hairpin 35 and 48 Loops of SSU rRNA Gene

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Abstract—A rare nucleotide substitution was found in the evolutionarily conserved loop of hairpin 35 of the 18S rRNA gene of marine free-living nematode, *Trefusia zostericola* (Nematoda: Enoplida). The same substitution was found in all the marine Enoplida studied but not in other nematodes. Such a molecular synapomorphy indicates that marine enoplids are more closely related to *T. zostericola* than to freshwater Triplonchida. Maximum parsimony, neighbor-joining, and maximum likelihood analyses of complete nucleotide sequences of the gene, with the heterogeneity of nucleotide sites in evolution rates taken into account, support this conclusion. Hence, the hypothesis of particular primitiveness of Trefusiidae among nematodes should be rejected. Phylogenies based on molecular data support the morphological reduction of metanemes in Trefusiidae. Alongside with the unique change in hairpin 35 loop among marine Enoplida (including *T. zostericola*), hairpin 48 is also modified by a rare transversion which could be found among Mesorhabditoidea nematodes, in related genera *Pelodera*, *Mesorhabditis*, *Teratorhabditis*, *Parasitorhabditis*, *Crustorhabditis*, and *Distolabrellus*, and in 11 orders of Rhodophyta. Rare mutations in hairpins 35 and 48 tend to be fixed correlatively in evolution and could be found in all the Acanthocephala species. X-Ray data show that these regions (H31 and H43, in alternative nomenclature) are spatially brought together in native ribosomes. The nature and distribution of molecular autoapomorphies in phylogenetic trees of high-rank taxa are discussed.

Key words: 18S rRNA gene, molecular phylogeny, Nematoda, Adenophorea, Enoplida, Trefusiidae, long-branch attraction (LBA) effect

INTRODUCTION

The breakthrough in *Caenorhabditis elegans* genome sequencing placed it among organisms most profoundly studied by molecular methods, and made its genome a milestone in comparative studies of genes of other multicellular animals. In addition, *C. elegans* is one of the model objects in embryology. The influence of mutations of some genes is intensely studied in relation to programmed cell death during embryogenesis, to development of the nervous system, and in other aspects. To understand which of these properties are typical of *C. elegans* and which could be found in other animal taxa differently related to *C. elegans*, new taxa are to be studied, especially those belonging to Nematoda [1]. Nowadays, large-scale investigations are being carried out on some animal and plant nematode parasites (*Brugia malayi*, *Onchocerca volvulus*, *Ancylostoma caninum*, *Meloidogyne incognita*) and free-living nematode, *Pristionchus pacificus*. Numerous documents on the genome structure of these species are kept in GenBank. Knowledge of the phylogenetic relatedness of nematodes could help select species for further nucleotide sequence studies. In this respect, free-living marine nematodes of the Enoplida order are of special interest. Basing on the morphological characters of these animals, a hypothesis has been suggested that this group is the most primitive among nematodes [3, 4]. Cladistic analysis has isolated from Enoplida a paraphyletic residue that was combined in order Trefusiida [5]. However, no synapomorphies in anatomy and fine structure of these animals have been revealed [5], hence cladistic analysis attributes them to the most primitive nematodes. Previous analyses also placed Trefusiidae at the basis on the nematode evolutionary tree [6–8] because Trefusiidae do not have well-developed characters of morphological specialization [4–6].

The aim of the present study was to test the hypothesis of the ancestral origin of Trefusiidae employing molecular data. Molecular synapomorphies were discovered in *Trefusia zostericola*, as well as in Enoplida species. This observation contradicts the hypothesis of particular evolutionary primitiveness of Trefusiidae species.

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EXPERIMENTAL

DNA was isolated from ethanol-fixed *Trefusia zostericola* specimen collected in the White Sea Bay of Kandalaksha. PCR-amplified fragments of 18S rRNA genes were obtained using primers described earlier [9], purified electrophoretically and cloned in pBluescript KS+ (Stratagene) linearized with *Eco*RV. Nucleotide sequences of both strands were determined using the *fmol* DNA Sequencing System (Promega) with universal primers to the vector part of plasmid and a set of internal primers complementary to conserved regions of the sequenced gene. The resulting sequence (GenBank AF329937) was a consensus of four completely and two partially sequenced clones. The numbers of GenBank sequences used in phylogenetic analysis are given in the figures. Sequences were aligned manually using the model of 18S rRNA secondary structure given in [10]. Alignments can be obtained on request (petr@bioevol.genebee.msu.su). Complete sequences were used in analyses except the V9 hypervariable region (apical part of hairpin 49). MP, NJ (PHYLIP [11]), and ML (fastDNAml, version 1.2.2 [12]) methods were employed to construct phylogenetic trees. Bootstrap analysis (1000 resamplings for MP and NJ trees and 20 resamplings for ML trees) was used to assess the statistical validity of internal nodes [13]. For ML trees, an alternative approach was used, taking into account numerous suboptimal trees which did not differ from the minimal tree by the Kishino–Hasegawa criterion [14]. When phylogenetic trees were constructed with regard to differences in the evolutionary rate of site-specific substitution, parameters of gamma-distribution were calculated using PUZZLE 4.0 [15] and TN model [16] with six categories of sites differing in the rate of substitution accumulation. The same program and model were employed in four-cluster mapping [17].

RESULTS AND DISCUSSION

Nucleotide sequences of the 18S rRNA gene of representatives of all the main evolutionary lineages of Nematoda contained in GenBank were analyzed. Among them, the set of Enoplida sequences was exhaustive to date. Representatives of aschelminths (nematophores, priapulids, gastotrichs, and rotifers) and more distant evolutionary lineages, annelids and cnidarians, were used as outgroups.

All the methods of tree construction employed gave similar results demonstrating that nematodes could be separated into four main groups (Fig. 1). The following largest clades, corresponding to (i) the subclass Chromadoria (including Rhabditia = Secernentea, Plectida, Monhysterida, Desmodorida, Chromadorida, and some other orders absent from Fig. 1), (ii) Dorylaimia (including Dorylaimida, Mononchida, Mermithida, and Trichinellida orders), (iii) Triplonchida (including Tobrilina and Diphtherophorina), and, (iv) Enoplida (including marine representatives of the order) have the highest bootstrap support. The composition of these clades differs from the conventional one, but this result is in accord with data obtained earlier in molecular and embryological phylogenetic studies [18–20]. The sequence of the 18S rRNA gene of *Trefusia zostericola* with high bootstrap support at all the trees belongs to the same cluster as those of other marine Enoplida, except for the MP and NJ trees constructed without correction for the site-specific heterogeneity of evolution rates (Fig. 1).

To check the reliability of placement of *T. zostericola* in the Enoplida cluster, the ML method of phylogenetic mapping was employed [17]. The same set of species excluding outgroups was subdivided into four groups (Enoplida, Triplonchida, Dorylaimia, and Chromadoria) corresponding to the monophyletic clades at the tree (Fig. 1). *T. zostericola* was taken as a separate group to verify its position at the tree. For each of the five possible quartets of taxa, support values were calculated for each of the three possible topologies using the TN model corrected for the heterogeneity of sites in the rate of evolution. The results of this analysis are presented in the table. It is evident that the highest support in all the cases is observed in topologies uniting *T. zostericola* with Enoplida (67.7, 66.3, and 62.8% in different combinations of the taxa quartets). All the alternative combination of *T. zostericola* with in other taxa had much lower support.

DNAPARS (PHYLIP package) allows deducing stepwise changes in sequences for each node of the maximum parsimony tree constructed with the aid of this program. The set of the inferred Enoplida apomorphies (including *T. zostericola* and excluding Triplonchida) contains 55 characters. Comparison of the corresponding regions in an extended set of 18S rRNA sequences shows that 53 of them are located in the evolutionarily variable regions of the gene. For 43 characters, variations were observed even among five analyzed sequences of marine Enoplida. In other words, fixation of nucleotide substitutions at these sites took place repeatedly during evolution. For 11 traits identical for five Enoplida, frequencies of homoplastic substitutions among representatives of other nematodes could differ. The only A $\longrightarrow$ G transition (site 1195 in *Enoplus brevis* sequence) is located in the conserved region of the gene. Among nematodes, it is characteristic only of Enoplida (excluding Triplonchida) and can be found in all the species studied (Fig. 2).

A transition typical of enoplids is localized in the single-stranded loop of the rRNA hairpin 35 (Fig. 2). The primary structure of this loop is highly conserved not only among nematodes, but also among multicellular animals in general. For example, only traces of

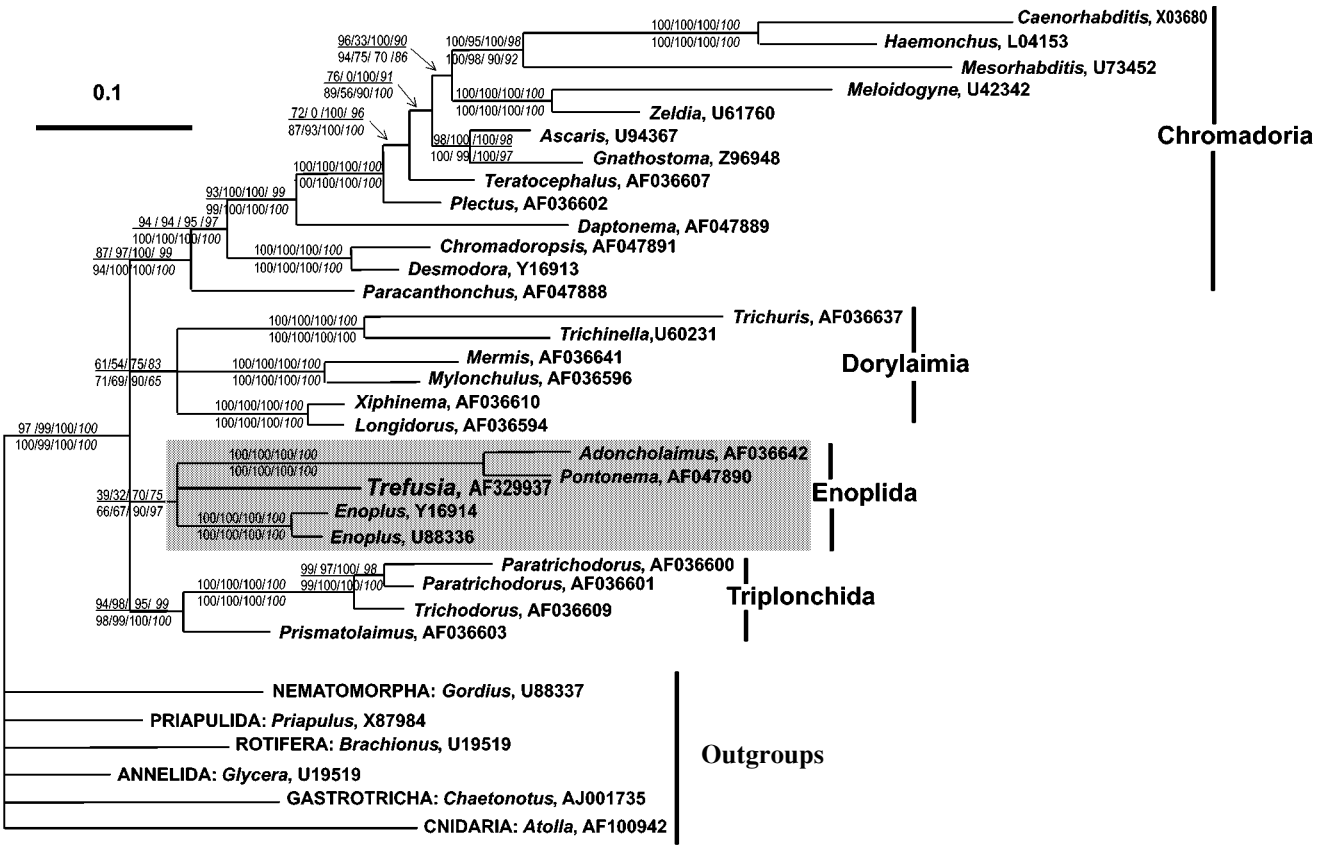

Fig. 1. Position of *Trefusia zostericola* at the nematode phylogenetic tree. Tree construction method-dependent nodes or those having low bootstrap support are collapsed. Branch lengths were calculated using the ML method and PUZZLE program, taking into account heterogeneity of the site-specific rate of evolution and employing gamma-distribution with six categories of substitution rate, TN model. Figures at the nodes give (from left to right) the bootstrap support values at consensus tree for maximum parsimony, distance NJ, and maximum likelihood trees (for 1000, 1000, and 20 replicates, respectively); given in italics are frequencies of grouping in the set of suboptimal ML trees (in %) which are not statistically different from the optimal tree by the Kishino–Hasegawa criterion. The analyzed set included 287 out of 17,560 examined trees of various topologies (when unweighted data were analyzed) and 72 trees out of 6234 when site-specific heterogeneity of the substitution rate was taken into account. Bootstrap support values obtained without such correction are given above the line, and the corrected values below it.

three fixed transitions in the corresponding site were detected beyond Enoplida among 880 aligned sequences of the metazoan 18S rRNA genes [10] (http://rrna.uia.ac.be/ssu). The same G residue was found in *Acheta domesticus* (GenBank X95741), in one out of three dicyemid sequences, and in all 21 sequenced 18S rRNA genes of Acanthocephala species from all three classes of this phylum. When we employed the BLAST program [21] in GenBank study and examined aligned 18S rRNA gene sequences of metazoans, we found two analogous transitions, one in *Trypodendron lineatum* (beetle, GenBank AF250076) and in an aberrant myxozoan PKX 52134 (GenBank U79623). Outside Metazoa, such a transition is also rare in many groups: according to the data accumulated in the Antwerp database [10], it was found only in one species among 1040 aligned sequences of fungal rRNA genes, and in one species out of 301 species of Alveolata. It was found neither among 211 species of red algae, nor among 122 species of heterokonta, etc.

Aligned fragments shown in Fig. 2, in addition to the Enoplida synapomorphy in loop 35 registered by DNAPARS, have eight other "apomorphies." Most of them are localized in variable sites which were repeatedly substituted in the genes under study. Transversion G $\longrightarrow$ T in hairpin 48 loop (site 1601 in *Enoplus brevis* sequence) looks different. This is an evolutionarily conserved region similar to hairpin 35. Only in two nematode taxa, Enoplida and distantly related Mesorhabditoidea, the nucleotide in the corresponding site of loop 48 is substituted by thymine residue. Such substitution could be registered practically in every species of these taxa. Among Mesorhabditoidea, it was found in 15 sequences belonging to species from the genera *Pelodera* (except two unpublished sequences out of six known), *Mesorhabditis*, T*eratorhabditis*, *Parasitorhabditis*, *Crustorhabditis*, and *Distolabrellus* (literature data included) [22–24].

They are all presented in GenBank and at http://www.nyu.edu/projects/fitch/fresearch/fsystemat/frystemat.html. Summing up, although this character is obviously a homoplastic one for Enoplida and Mesorhabditiodea, it might serve as a phylogenetic marker separating these two groups from related clades.

When G ⟶ T transversion in hairpin 48 loop was searched for outside nematodes, it proved to be rare. For example, according to the Antwerp set mentioned above [10], it is present only in one fungal sequence out of 1040, in none of 122 stramenopiles sequences, in seven out of 860 sequences of green plants (algae), four of which belong to Ulvales, etc. Rhodophyta appear to be an exception to this rule: the thymine residue at the corresponding position is abundant, 131 sequences out of 211. Differentiated registration of distribution of this character among Rhodophyta revealed that among Bangiophyceae and in seven orders of Florideophyceae the character is plesiomorphic, and only among representatives of 11 orders of Florideophyceae it is apomorphic and could have resulted from a single evolutionary event. Among Metazoa, G ⟶ Y transition was detected in one species of hexactinellids, two species of Hydrozoa, two species of Cladocera, one species of Ostracoda, and one species of Decapoda, as well as in two unrelated species of turbellaria, one species of Aleyrodidae, in *Acheta domesticus*, in myxozoan PKX 52134, and in all acanthocephalids. In the latter three taxa, the A ⟶ G transition in hairpin 35 loop was also registered. Hence a tendency to concerted changes in the loops of hairpins 35 and 48 could be suggested, although this correlation is not absolute.

The relatedness of the four main nematode clades (Enoplida, Triplonchida, Dorylaimia, and Chromadoria) remains obscure. Four taxa can produce rooted trees of 15 topologies, many of which are realized by different tree construction methods. Depending on the tree construction method, the highest bootstrap supports are obtained for alternative options of the initial bifurcation (not shown). Hence, inclusion of *T. zostericola* 18S rRNA gene sequence into one of the clusters would not help reject the primordial position of Trefusiidae. The rate of evolution of the 18S rRNA gene in nematode lineages differs greatly. Sequences of many rhabditid nematodes, e.g., of *Pelodera strongyloides*, diverged from the "standard" sequence to the greatest extent and might be an excellent example [22]. In some high-rank nematode taxa (Trichinellida, Trichodoridae, Oncholaimidae) all the 18S rRNA gene sequences are fairly different from standard ones. Sequences with many substitutions tend to occupy wrong positions at the trees even if special phylogenetic programs based on algorithms formally unrelated to the molecular clock hypothesis are exploited [25–27]. This might account for a low reliability of bootstrap support calculated for *Trefusia* + Enoplida group at MP and NJ trees obtained disregarding site heterogeneity. For nematodes, it has been demonstrated experimentally that the results of tree construction depend on the selection of species sets [28, 29]. If one had not applied cladistic analysis of molecular traits, one could scarcely prove that the *T. zostericola* + Enoplida cluster is not an artifact of the ML method of tree construction or of the correction based on gamma-distribution. Reliability of *Trefusia* + Enoplida group is even better proved by the presence of a synapomorphy in hairpin 35, which is plesiomorphic in other nematodes (Fig. 2). The direction of evolution in this fragment of the gene, i.e., apomorphy of marine Enoplida, becomes evident when large samples of "outgroups" of animals belonging to other groups are analyzed, including unicellular eukaryotes. Evaluating the probability of the potential substitutions in hairpins 35 and 48 as being equal to 10^{-2} each according to their frequencies in the analyzed sets, we estimate the probability of independent origin of two "enoplid" characters as 10^{-4}. Therefore, such an event could hardly stochastically occur in the set of $2 \cdot 10^2$ sequences of Nematoda 18S rRNA gene sequences known to date. It should be mentioned, however, that in classical NJ analysis without correc-

Trefusia position assessed by maximum likelihood mapping

Combination of four taxa[a]	Alternative topologies[b]	Support[c]
I *Tref, Enop, Dory, Trip*	(*Tref, Enop*), (*Dory, Trip*)	67.7
	(*Tref, Dory*), (*Enop, Trip*)	18.8
	(*Tref, Trip*), (*Enop, Dory*)	13.5
II *Tref, Trip, Dory, Chro*	(*Tref, Trip*) (*Dory, Chro*)	23.7
	(*Tref, Dory*) (*Trip, Chro*)	33.5
	(*Tref, Chro*) (*Trip, Dory*)	42.8
III *Tref, Enop, Trip, Chro*	(*Tref, Enop*) (*Trip, Chro*)	66.3
	(*Tref, Trip*) (*Enop, Chro*)	1.0
	(*Tref, Chro*) (*Enop, Trip*)	32.7
IV *Tref, Enop, Dory, Chro*	(*Tref, Enop*) (*Dory, Chro*)	62.8
	(*Tref, Dory*) (*Enop, Chro*)	8.5
	(*Tref, Chro*) (*Enop, Trip*)	17.5
V *Enop, Trip, Dory, Chro*	(*Enop, Trip*) (*Dory, Chro*)	51.6
	(*Enop, Dory*) (*Trip, Chro*)	31.8
	(*Enop, Chro*) (*Dory, Trip*)	16.6

[a] Tref, *Trefusia*; Enop, Enoplida; Trip, Triplonchida; Dory, Dorylaimia; Chro, Chromadoria.

[b] Triplets of alternative topologies of rootless tree possible for four taxa.

[c] Proportion (%) of possible quartets supporting a given topology.

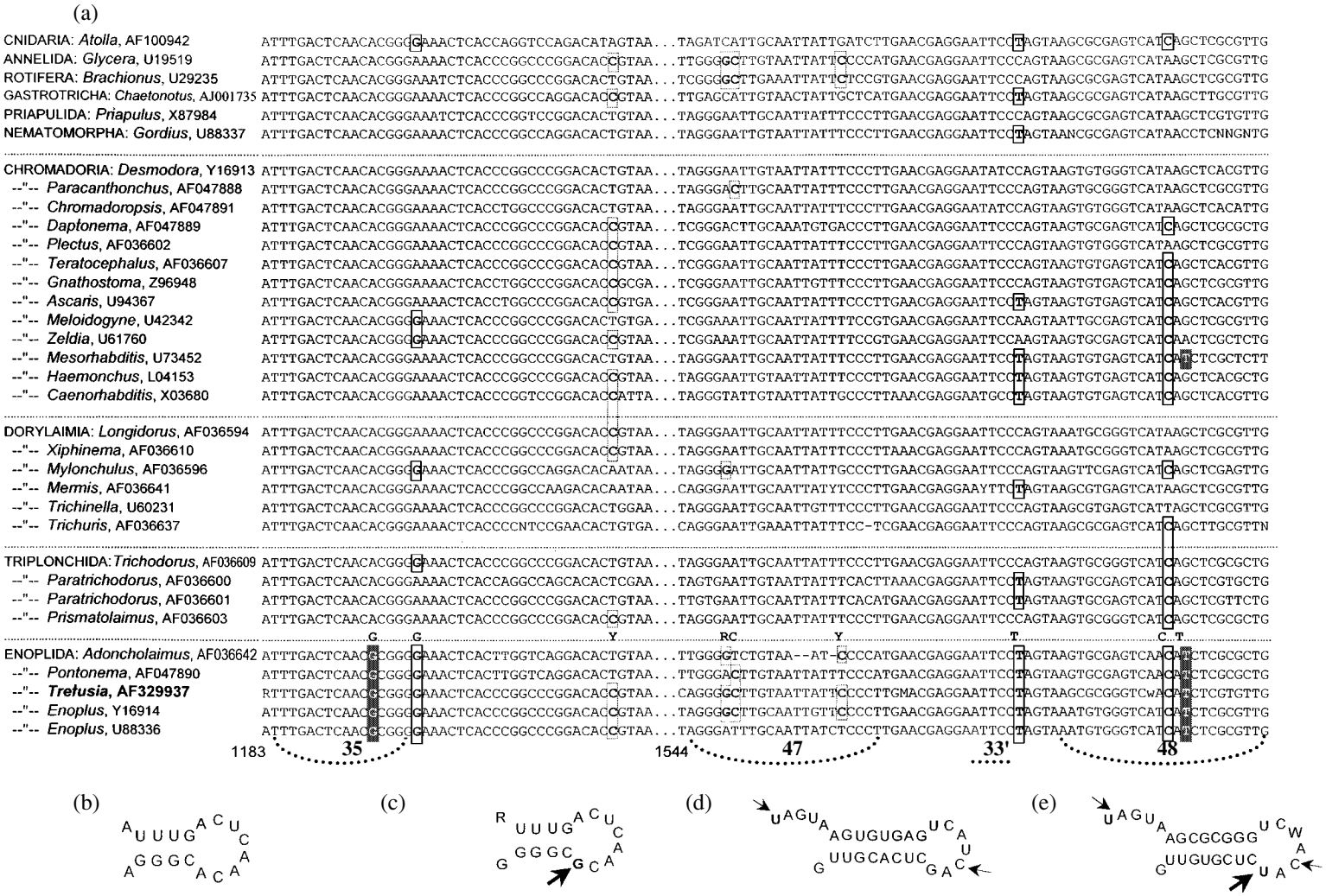

Fig. 2. Localization of synapomorphic molecular traits in 18S rRNA genes of marine Enoplida. (a) Fragments of aligned gene sequences corresponding to 18S rRNA regions of helices 35 and 48. Broken lines separate four firmly established nematode clades (see the text). Presumed synapomorphies of *Trefusia zostericola* and other marine Enoplida, deduced by the DNAPARS program (PHYLIP package), are marked at the lower broken line. Broken lines frame identical residues in the detected highly variable sites, and solid ones frame conserved residues. Highly conserved sites are given at dark background. Dotted arcs and bold figures show canonical regions of double helices in rRNA. Only generic names of species and GenBank numbers are given. Sequences are enumerated as in *E. brevis* rRNA. Bottom, secondary structures of hairpin 35 of (b) *Caenorhabditis elegans* and (c) *T. zostericola* and hairpin 48 of (d) *C. elegans* and (e) *T. zostericola*.

tion for site heterogeneity by the evolution rate, 68% of bootstrap replicates do not support the *Trefusia* + Enoplida clade. Thus, the results of cladistic analysis are not only in accord with the results of conventional analysis of complete sequences of the gene, but appear to be a more reliable proof of the monophyly of Trefusiidae + marine Enoplida. Hence we reject the ancestral position of Trefusiidae among nematodes.

A question arises whether the result obtained is applicable to all trefusiids. This may be true if their monophily is strictly proved. Five families of substantially different nematodes (Trefusiidae, Simpliconematidae, Lauratonematidae, Xenellidae, and Onchulidae) having one common "negative" character (absence of metanemes, specific subcuticular proprioreceptors characteristic of Enoplida) were united in the order Trefusiida [5]. However, the phylogenetic result obtained in this study testifying that marine Enoplida are closely related to *T. zostericola* might be the basis for revision of metaneme evolution. As all the Enoplida and some Tobrilidae (Triplonchida) possess metanemes, they should have been be present in the common ancestor of these groups [5, p. 75]. Hence the lack of metanemes in Trefusiida could be explained by their loss in evolution, and the simplicity of the group formerly considered as an ancestral one might be based on partial morphological reduction. A consequence of the above discussion is that the absence of metanemes is a Trefusiidae synapomorphy, and not symplesiomorphy, as suggested in [5]. However, we cannot exclude that these structures were reduced independently. Moreover, sometimes they are difficult to identify. Therefore, the phylogeny of other families of Trefusiida should be considered separately, and data on the structure of hairpin 35 might help solving this problem.

Nowadays, molecular data are still insufficient to discuss the position of Trefusiidae within Enoplida. Relationships of Trefusiidae, Enoplidae, and Oncholaimidae are presented in Fig. 1 as an unresolved trifurcation, because the position of *T. zostericola* at the 18S rRNA gene tree depends on the algorithm of tree construction. It might be severely distorted because of the length of branches leading to Oncholaimidae. However, the conclusion that the lack of metanemes in Trefusiidae is a secondary character induces one to look for their relatives among Enoplida families having metanemes, e.g., among Trypiloididae. The latter were supposed to be related to Trefusiidae basing on similarities in the structure of cephalic setae of the second circle in some species of Trefusiidae and Trypiloididae [30].

The significance of highly conserved substitutions in hairpins 35 and 48 of the marine Enoplida is more difficult to interpret than the evolution of metanemes. As in many similar cases of application of molecular markers, one might suppose that in native ribosomes such residues participate in RNA–ligand (protein or RNA) interactions [31]. Hairpin 35 loop (hairpin 31 in alternative nomenclature) is of great functional importance in the ribosomal P-site [32]. X-Ray analysis of 30S ribosome subunits of *Thermus thermophilus* at 3 Å resolution reveals spatial closeness of hairpins 35 and 48 (31 and 43 in alternative nomenclature) although the latter hairpin is not a member of the P-site [32, 33]. At least one protein (S9) appears to bind to RNA at two homologous hairpins in bacteria [33]. However, it remains obscure which residues and of which molecules serve as G1195 and U1601 ligands in *Enoplus brevis* and other Enoplida, and why these residues substituted the ancestral A and G residues. The rare occurrence of substitutions in these positions and the long-lasting evolutionary stability of such a change if it takes place, e.g., in all the Enoplida or the whole Acanthocephala phylum, support the hypothesis that such changes might be accompanied by compensatory changes in ligand structures, and thus evolution might follow the punctuated equilibrium scenario [34, 35]. The observed concertedness of changes in hairpins 35 and 48 also needs explanation. It cannot be due to direct complementary interaction of these residues, and it is not strict enough. As to the rarer A $\longrightarrow$ G transition in hairpin 35, in only about half of the cases it is accompanied by G $\longrightarrow$ Y transversion in hairpin 48. Although being functionally obscure, apomorphic changes in hairpins 35 and 48 are good markers for identifying new sequences of 18S rRNA genes of marine Enoplida. These markers allow one to obviate mistakes caused by long branch attraction. The same is true for hairpin 48 characteristic of Mesorhabditiodea. The nucleotides mentioned above could be the targets for specific primers in these nematode taxa.

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