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Short Communication

Taxonomic implications of molecular studies on Northern Brazilian Teredinidae (Mollusca: Bivalvia) specimens

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Abstract

The current taxonomy of the Teredinidae (shipworms) is wholly based on morphology and up to now no molecular studies of the phylogeny of this group have been published. In the present study the relationships between four genera of the subfamilies Teredininae and Bankiinae were established and the efficiency of the 16S rRNA gene in characterizing four Teredinidae species was tested. Phylogenetic trees support the grouping of *Bankia fimbriatula* with *Nausitora fusticula* and of *Neoteredo reynei* with *Psiloteredo healdi*, but the genetic distances do not justify the classification of these species into two distinct subfamilies. The results show that *B. fimbriatula*, *N. reynei* and *P. healdi* specimens from the coast of the Brazilian state of Pará have five distinct 16S rRNA haplotypes, with one *N. reynei* haplotype differing from the other haplotypes in respect to at least seven sequences sites, indicating the existence of two very distinct sympatric lineages.

Key words: Teredinidae, rRNA16S, phylogeny, mtDNA.

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Members of the family Teredinidae (Mollusca, Bivalvia), commonly known as shipworms, are highly specialized bivalves mollusks which have a greatly reduced shell adapted for boring into wood and which produce a calcareous wall along the tunnels where they live (Turner, 1966). The Teredinidae family is one of the more difficult groups to classify and its taxonomy was in a chaotic state until Turner's classification based on anatomical studies, life histories and the structure and development of the pallets was published (Turner, 1966; Nair and Saraswathy, 1971) which divided the teredinids into six main groups (Table 1), further arrangement of the six groups resulting in the division of the family into three subfamilies: Kuphinae (group I); Teredininae (groups II, III and IV); and Bankiinae (groups V and VI). Teredinidae genera and species are identified based mainly on the shape of their siphons and the pallets (Turner, 1966), with the genus *Neoteredo* having the striking and unique feature of a pair of large lappets on the dorsal surface of the body just anterior to the siphons (Turner, 1966).

There are no phylogenetic studies of teredinids based on molecular data and only a few studies using biochemical analysis, Cole and Turner (1977, 1978) having used allozyme data to study relatedness between selected species within the families Pholadidae and Teredinidae while Hoagland (1986) used allozyme phenotypes to investigated the intrapopulation genetic variation and genetic distance between seven teredinid and pholadid species in the bivalve superfamily Pholadacea.

The lack of molecular data on teredinids led us to study the phylogenetic relationships based on molecular data of four Teredinidae genera, two (*Bankia* and *Nausitora*) currently placed in the Bankiinae subfamily and two (*Neoteredo* and *Psiloteredo*) in the Teredininae subfamily.

The *Neoteredo reynei*, *Bankia fimbriatula* and *Nausitora fusticula* specimens were collected from saltwater and *Psiloteredo healdi* from freshwater (Table 2) and identified according to Turner (1966). Tissue samples were collected from the anterior or posterior adductor muscle

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Table 1 - Teredinidae as grouped by Turner (1966).

Group	Genus	Naming author and date
I	*Kuphus*	Guettard 1770
II	*Bactronophorus*	Tapparone-Canefri 1877
	Neoteredo	Bartsch 1920
	Teredothyra	Bartsch 1921
	Dicyathifer	Iredale 1932
III	*Uperotus*	Guettard 1770
	Teredora	Bartsch 1921
	Psiloteredo	Bartsch 1922
IV	*Teredo*	Linnaeus 1758
	Lyrodus	Gould 1870
V	*Nototeredo*	Bartsch 1923
VI	*Bankia*	Gray 1842
	Nausitora	Wright 1864
	Spathoteredo	Moll 1928

and the DNA extracted using the protocol of Sambrook *et al*. (1989). Mitochondrial DNA sequences of the region 16S rRNA (about 456 bp) were determined by direct sequencing of polymerase chain reaction (PCR) amplified fragments using the L2510 5'-CGCCTGTTTATCAAAA ACAT-3' forward primer and the H3059 5'-TTTCCCCG CGGTCGCCCC-3' reverse primer designed by Palumbi *et al*. (1991) and Farias *et al*. (1998). Reactions were denatured at 94 °C for 3 min and subjected to 25 cycles of one minute at 94 °C, one minute at 50 °C and two minutes at 72 °C with a final extension step at 72 °C for five minutes. Amplification products were purified using ExoSap IT (Amersham-Pharmacia Biotech. Inc., Piscataway, NJ, USA). The DNA sequences were determined using dye terminator cycle sequencing reactions that were subsequently loaded onto an automatic sequencer (Applied Biosystems model 377) according to the manufacturers protocols. The nucleotide sequence data from the partially sequenced 16S rRNA produced in our study was deposited in GenBank under accession numbers AY633493 to AY633509.

Initial sequence alignments were performed using the BioEdit (Hall, 1999) and sequence alignment Clustal W (Thompson *et al*., 1994) programs. The nucleotide frequencies and the transition/transversion ratio were obtained using the MEGA 2 program (Kumar *et al.*, 2001). The saturation test was performed by plotting transitions and transversions against K2P (Kimura, 1980) distances using the data analysis in molecular biology and evolution (DAMBE) program (Xia and Xie, 2001). In order to identify the evolutionary model that best fitted the data the aligned sequences were tested with the Modeltest program version 3.06 (Posada and Crandall, 1998). The divergence matrix was generated using the phylogenetic analysis using parsimony (PAUP) 4.0 program beta version (Swofford, 2002) based on the model established by the Modeltest program. Maximum-parsimony (MP), neighbor joining (NJ) and maximum-likelihood (ML) analyses were performed with the PAUP 4.0 program using an heuristic search. Gaps were treated as missing data. The robustness of the phylogenetic hypothesis obtained was tested by bootstrapping

Table 2 - Species, collection sites and specimen codes of the teredinid species used in this study. The Itajaí collection site was in the Brazilian state of Santa Catarina, all the other sites being in Pará state, Brazil.

Species	Species code	Collection site	Site code	Specimen code
Tereduninae subfamily				
Neoteredo reynei	Nr	São João de Pirabas	Pi	NrPi50, NrPi12, NrPi62
		Salvaterra, Marajó Island	St	NrSt105, NrSt106
		Salinas	Sa	NrSa9
		Canela Island, Bragança	Ca	NrCa3
		Furo Grande, Bragança	Fg	NrFg21, NrFg03
Psiloteredo healdi	Ph	Furo Cumbu, Belém;	Fc	PhCu192, PhCu193, PhCu201
		Salvaterra	St	PhSt101, PhSt103, PhSt113, PhSt114, PhSt115, PhSt123, PhSt171
Bankiinae subfamily				
Bankia fimbriatula	Bf	Furo Grande	Fg	BfFg46, BfFg59
		Tamatateua, Bragança	Ta	BfTa33, BfTa37, BfTa43
Nausitora fusticula	Nf	Itajaí	It	NfIt01

(Felsenstein, 1985) using 2000 replicates for the MP analysis and 1000 replicates for the NJ and ML analyses, bootstrap values equal to or greater than 90% were considered statistically significant. Bremer Decay indices were obtained with the SEPAL program (Salisbury, 1999, 2000).

The outgroup was *Martesia striata* (Pholadidae) but alignment was only possible for 350 bp, *i.e.* the first 190 bp and interspersed fragments. The parsimony tree including *M. striata* (score: 193) showed a trichotomy involving *Bankia*, *Nausitora* and *Neoteredo-Psiloteredo*. Since the outgroup was not informative in determining the basal genus and alignment was only possible when about 100 bp were removed from the data set we decided to exclude *M. striata* from our analysis. The fragments obtained were composed of 456 sites after alignment without the outgroup and showed 121 parsimony informative sites. It was necessary to introduce 7 insertions and deletions (indels) to align the sequences. The average base frequencies were adenine = 0.260, cytosine = 0.148, guanine = 0.287 and thymine = 0.305 and the transition/transversion rate as calculated by the Mega 2 program was 1.4. The number of transitions and transversions were plotted as a function of p-distance and no saturation was observed. The sequences showed that there are at least five 16S rRNA haplotypes for *N. reynei* (N1 to N5), *P. healdi* (P1 to P5) and *B. fimbriatula* (B1 to B5), the intraspecific haplotypes identified for these three species differing by one to four base pairs, except for one *N. reynei* haplotype (N5) which presented at least seven differences when compared with the other *N. reynei* specimens (haplotypes N1 to N4).

The best-fit maximum-likelihood model for the 25 specimens selected by the Modeltest program was the Hasegawa-Kishino-Yano model (Hasegawa *et al*., 1985), considering gamma (G) and the distribution shape parameter (HKY+G). The distance matrix showed intraspecific divergencies of 0 to 0.0179 for *N. reynei*, 0 to 0.0091 for *P. healdi* and from 0.0024 to 0.0055 for *B. fimbriatula*. The intergeneric distances showed similar values, with *Neoteredo* and *Psiloteredo* showing values ranging from 0.1943 to 0.2214, *Bankia* and *Nausitora* from 0.1815 to 0.2102, *Psiloteredo* and *Nausitora* from 0.1857 to 0.2098, *Psiloteredo* and *Bankia* from 0.2034 to 0.2551, *Neoteredo* and *Nausitora* from 0.2245 to 0.2376 and *Neoteredo* and *Bankia* from 0.2356 to 0.3205. Comparison of divergence values showed that the N5 haplotype, rather than the N1-N4 haplotypes, is more closely related to *Psiloteredo*. The trees obtained in this study using different methods (MP, ML and NJ) showed the same general topology (Figure 1). A heuristic search recovered a single most parsimonious tree (score: 195). *Nausitora* was closely related to *Bankia* with significant bootstrap values of 90%, (MP), 92% (ML) and 96% (NJ) and a Bremer Decay index of 7. We found that *Psiloteredo* grouped with Bankiinae (bootstrap = 100% for all trees; Bremer Decay index = 29), then the *Neoteredo* haplotype N5 (specimens NrSt105, NrFg03 and NrPi62) joined *Psiloteredo*-Bankiinae subfamily group with bootstraps values of 91% (MP), 82% (ML) and 99% (NJ) and a Bremer Decay index of 3, followed by *Neoteredo* haplotypes N1 to N4. As the trees were not rooted it was not possible to establish a basal genus (see Figure 2).

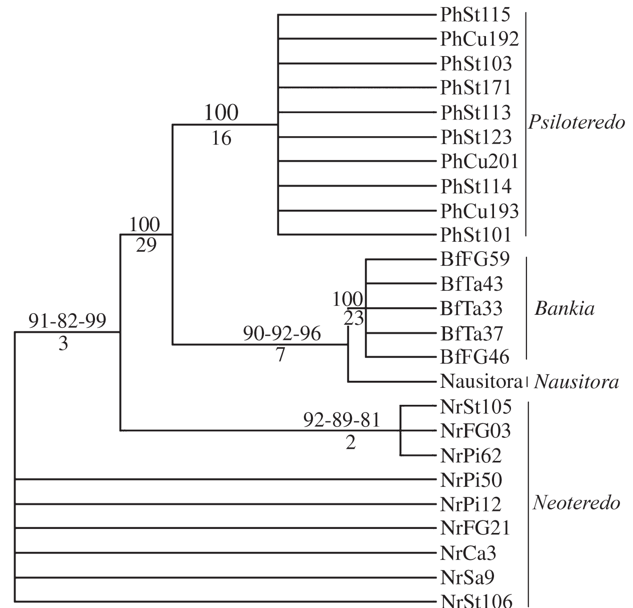

Figure 1 - Phylogenetic consensus tree obtained using maximum parsimony (MP), maximum likelihood (ML) and neighbor joining (NJ). Numbers above the branches indicate the bootstrap values (MP (left), ML (center) and NJ (right)). A single number above the branches indicates that the three methods gave the same bootstrap value. Numbers below the branches show Bremer Decay indices. Species codes: Ph = *Psiloteredo healdi*; Bf = *Bankia fimbriatula*; Nr = *Neoteredo reynei*. Collection site codes: St = Salvaterra; Cu = Cumbu; FG = Furo Grande; Ta = Tamatateua; Ca = Canela Island; Sa = Salinópolis; Pi = São João de Pirabas.

A question which needs to be addressed in teredinid systematics is whether or not *Neoteredo reynei is* a single species. All trees generated showed a clear division of *N. reynei* into two groups (haplotypes N1 to N4 and haplotype N5) although only the maximum parsimony method showed significance in the bootstrap test. The distance matrix showed that there were high intraspecific divergence

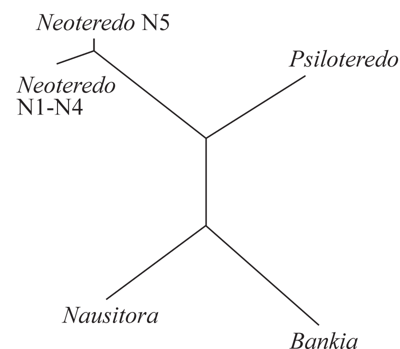

Figure 2 - Relationships between four Teredinidae genera. N1 to N5 represent different *Neoteredo reynei* haplotypes.

values between the *N. reynei* specimens corresponding to the distance between the N5 haplotype specimens and specimens with the other haplotypes (N1 to N4). Jozefowicz and Ó Foighil (1998), studying the same fragment of rRNA16S in species of the genus *Ostrea* (Ostreinae, Bivalvia) obtained Kimura 2-parameter distance values ranging from 0.0416 to 0.0923 and a value of 0.0712 was found between two species of *Dendostrea* (Lophinae, Bivalvia). Using the same region and the Jukes-Cantor matrix, Stepien *et al*. (1999) observed that the distance value between *Dreissena bugensis* and *Dreissena polymorpha* (Dreissenoidea, Bivalvia) was 0.0790. In another study, Schneider and Ó Foighil (1999), also sequencing the 16S region and constructing the Jukes-Cantor matrix for species of two genera of the family Cardiidae (Bivalvia), found that two species of the genus *Hippopus* diverged by 0.0722 and the interspecific variation between six species of *Tridacna* range from 0.0401 to 0.1217. In order to compare these studies with our data we used the Mega 2 program to calculate the Kimura 2-parameter and the Jukes-Cantor genetic distance matrices for the specimens used in our study and found that the maximum values obtained in both matrices between the *N. reynei* N1 to N4 haplotypes was 0.0061 while between N5 and N1 to N4 the maximum value was 0.0246. When compared with other studies (Jozefowicz and Ó Foighil,1998; Schneider and Ó Foighil,1999; Stepien *et al*.,1999) the latter value (0.0246) was not high enough to suggest that the *N. reynei* N5 haplotype could be a different species, although the divergence values obtained between specimens with the N5 haplotype and the N1 to N4 haplotypes were at least twice those observed for the intraspecific distances of *Psiloteredo healdi* and 3.25 times those of *Bankia fimbriatula* which indicates the existence of significant variability among the *N. reynei* specimens. Mulvey *et al.* (1996) sequenced the 16S rRNA region of three species of the genus *Amblema* (*A. neislerii*, *A. plicata*, *A. ellioti*: Unionidae, Bivalvia) obtained from different rivers in the United States of America and identified six intrageneric haplotypes (A1 to A6). The A1 haplotype was present in all *A. neislerii* samples and A2 was found in all *A. elliotti* specimens, the A1 and A2 haplotypes differing in only one transversion which indicates that they only diverged recently. Four haplotypes (A3 to A6) were identified only in *A. plicata* and showed differences from the other two species in at least eight nucleotide sites. In the same study Mulvey *et al.* detected five haplotypes (M1 to M5) for two species of the genus *Megalonaias* which differed from each other in only one or two base pairs. According to Mulvey *et al.*, *Megalonaias boykiniana* and *Megalonaias nervosa* should be considered a single species (*M. nervosa*) because both presented the M1 haplotype. Our study showed one to four nucleotide differences in the intraspecific haplotypes of *P. healdi* and one or two for *Bankia fimbriatula*, however the differences for *N. reynei* were bigger, showing seven or eight distinct sites between N5 and the other haplotypes. The three *N. reynei* samples with the N5 haplotype came from distinct geographic localities (Salvaterra, São João de Pirabas and Furo Grande - Bragança) on the coast of Pará state, indicating a wide distribution within the same mangrove ecosystem shared with the other haplotypes. Based on the analysis of the haplotypes, we conclude that there are probably two cryptic species or at least two subspecies of *N. reynei*.

Another question is whether *Neoteredo healdi* should in fact be classified as *Psiloteredo healdi*. Turner (1966) reclassified *N. healdi* Bartsch 1931 as *P. healdi* because although *N. healdi* shows great similarity with *N. reynei* in terms of their pallets and shells the absence of lappets should place *N. healdi* in the genus *Psiloteredo*. Reis (1995) agreed with Turner (1966) and reiterated the importance of the presence of lappets as a defining morphological characteristic of the *genus Neoteredo*. In our present study, the analysis of the molecular distances supports the classification of Turner (1966) which placed *Psiloteredo healdi* and *Neoteredo reynei* in different genera.

Is the division of the Teredinidae into groups and subfamilies supported by molecular data? As outlined above, Turner (1966) divided the Teredinidae into six main groups and distributed the species among the three subfamilies Kuphinae, Bankiinae and Teredininae. We sequenced samples of two genera of Bankiinae (*Bankia* and *Nausitora*) and two genera of Teredininae (*Psiloteredo* and *Neoteredo*) and found that the divergence values did not support the grouping suggested by Turner (1966) because the genetic distance values between specimens of group II (*Neoteredo reynei*) and III (*Psiloteredo healdi*) were similar to those observed between *Bankia fimbriatula* and *Nausitora fusticula* both belonging to group VI. Although the phylogenetic trees showed topologies grouping the four genera according to Turner's subfamily classification (Figure 2), when we compare the divergence values of these four species they did not justify the division of *Bankia*, *Nausitora*, *Neoteredo* and *Psiloteredo* into two subfamilies. This view is supported by the work of Hoagland (1986) who used alloenzymes and Nei's distance matrix and obtained results indicating that biochemical studies did not support Turners subfamily classification because the divergence values obtained ranged from 0.71 to 1.09 between species of *Teredo* (Teredininae) and *Bankia* (Bankiinae) and from 0.57 to 1.04 between *Teredo* and *Lyrodus,* both Teredininae.

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