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Evolution of ITS1 rDNA in the Digenea (Platyhelminthes: Trematoda): 3′ End Sequence Conservation and Its Phylogenetic Utility

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Abstract. A comparison of ribosomal internal transcribed spacer 1 (ITS1) elements of digenetic trematodes (Platyhelminthes) including unidentified digeneans isolated from *Cyathura carinata* (Crustacea: Isopoda) revealed DNA sequence similarities at more than half of the spacer at its 3′ end. Primary sequence similarity was shown to be associated with secondary structure conservation, which suggested that similarity is due to identity by descent and not chance. Using an analysis of apomorphies, the sequence data were shown to produce a distinct phylogenetic signal. This was confirmed by the consistency of results of different tree reconstruction methods such as distance approaches, maximum parsimony, and maximum likelihood. Morphological evidence additionally supported the phylogenetic tree based on ITS1 data and the inferred phylogenetic position of the unidentified digeneans of *C. carinata* met the expectations from known trematode life-cycle patterns. Although ribosomal ITS1 elements are generally believed to be too variable for phylogenetic analysis above the species or genus level, the overall consistency of the results of this study strongly suggests that this is not the case in digenetic trematodes. Here, 3′ end ITS1 sequence data seem to provide a valuable tool for elucidating phylogenetic relationships of a broad range of phylogenetically distinct taxa.

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Introduction

In the course of an analysis of digenetic ITS1 sequences isolated from *Cyathura carinata* (Crustacea: Isopoda: Anthuridea), we found sequence similarities at the 3′ end of all ITS1 sequences of digeneans published so far. Such similarities indicate the presence of sufficiently conserved sequence information to perform phylogenetic analysis of the taxa of the digenetic subclass. However, previous studies in animals and plants uniformly suggest that ITS1 elements are far too variable for phylogenetic analysis above the species or genus level (Furlong and Maden 1983; Gonzalez et al. 1990; Wesson et al. 1993; Schlötterer et al. 1994; Vogler and DeSalle 1994; Baldwin et al. 1995; Morgan and Blair 1995; Schilthuizen et al. 1995; Baur et al. 1996; Goggin and Newman 1996; Miller et al. 1996; Tang et al. 1996). We are aware of only three exceptions. Within the genus *Melanoplus* (Insecta: Orthoptera), ITS1 sequences appeared to be too conserved for a phylogenetic analysis of different species (Kuperus and Chapco 1994). Chen et al. (1996) and also Beauchamp and Powers (1996) successfully employed ITS1 sequence data to study the phylogenetic relationships between corallimorpharian genera (Cnidaria: Anthozoa). It is therefore of particular importance to show that the observed sequence similarity across the whole digenetic subclass is due to identity by descent and there-

Table 1. Digenetic ITS1 sequences used in this study

Abb.[a]	Species name	Systematic classification[b]	Length[c]	GB No.[d]
Dsy	*Dolichosaccus symmetrus*	Plagiorchiformes	525	L01631
Dsp	*Dolichosaccus* sp.	″	868	L01630
Oba	*Opechona bacillaris*	Lepocreadiiformes	620+?	Z29504
Lel	*Lepidapedon elongatum*	″	699+?	Z29497
Lra	*L. rachion*	″	707+?	Z29501
Sma	*Schistosoma mattheei*	Strigeiformes	618	Z21718
Sha	*S. haematobium*	″	457	Z21716
Eho	*Echinostoma hortense*	Echinostomatiformes	443	U58101
Eca	*E. caproni*	″	415	U58098
Etr	*E. trivolvis*	″	415	U58097
Epa	*E. paraensei*	″	415	U58100
Esp	*Echinostoma* sp.	″	414	U58103

[a] Abbreviation.
[b] Systematic classification according to Brooks et al. (1985, 1989).
[c] Length of the complete ITS1 elements in base pairs. ‘‘+?’’ indicates that ITS1 elements were only partially sequenced.
[d] GenBank accession number.

fore applicable to phylogenetic tree reconstruction procedures.

This work aims to test the phylogenetic utility of 3′ end ribosomal ITS1 sequences in digeneans by (i) a detailed a priori analysis of the data set to assess homology of nucleotide positions and phylogenetic signal consistency and (ii) a comparison of phylogenetic trees based on ITS1 sequences with hypotheses about trematode phylogeny as inferred from morphological data and the evolution of life-cycle patterns.

Materials and Methods

Materials and Molecular Techniques

Specimens of *Cyathura carinata* were collected in the Bay of Wismar (Baltic Sea, Germany), Flemhuder Meer (Baltic Sea–North Sea–Channel, Germany), Battenoord (Rhine-Maas-Schelde-Delta, The Netherlands), and the estuary of the Rio Cavado (Atlantic Sea, Portugal).

Genomic DNA was isolated using a modification of a protocol given by Winnepenninckx et al. (1993) and Gustincich et al. (1991). Isopod specimens were ground under liquid N_2, directly transferred into 400 μl of prewarmed (65°C) CTAB buffer [2% (w/v) CTAB, 0.1 *M* Tris–HCl, pH 8, 0.02 *M* EDTA, 1.4 *M* NaCl, 5% (v/v) β-mercaptoethanol] or DTAB buffer [6% (w/v) DTAB, 1.25 *M* NaCl, 75 m*M* Tris–HCl, pH 8, 75 m*M* EDTA], incubated for 1 h at 65°C, followed by extraction with 2 vol of chloroform:isoamylalcohol (24:1) and precipitation with ⅔ vol of 100% isopropanol. Amplification of ITS1 elements was performed via PCR in 50-μl reaction volumes [75 m*M* Tris–HCl, pH 9, 20 m*M* $(NH_4)_2SO_4$. 0.01% Tween 20, 1.5 m*M* $MgCl_2$, 0.8 m*M* dNTPs, 5 m*M* TMAC, 2 U of Goldstar DNA polymerase (Eurogentec, Seraing, Belgium), a 0.2 m*M* concentration of each primer (‘‘19,’’ 5′-CCAGTCGTAACAAGGTTTCCG; ‘‘4S1,’’ 5′-TCTAGATGCGTTCGAAGTGTCCATG), 1 μl of genomic DNA] and the following reaction profile: 5 min at 94°C, followed by 5 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 70°C, followed by another 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 70°C, and completed with a final extension period of 7 min at 70°C. PCR products were purified with the help of microcon microconcentrators (Amicon) and either directly sequenced or ligated into a T-tailed pGEM vector (Promega), followed by transformation of cells via electroporation (Bio-Rad). For positive clones, plasmid DNA was isolated with the help of the Wizard Minipreps DNA Purification System (Promega). DNA sequences of ITS1 elements were obtained via the dideoxy-chain termination method using the *fmol* DNA Sequencing System (Promega) and the Sequi-Gen GT System (Bio-Rad). For DNA sequencing, PCR primers and the following two internal ITS1 primers were used: ‘‘ITS1-1,’’ 5′-GAGCGCGCAGTTTCGTCCAATC; and ‘‘ITS1-2,’’ 5′-GGCCGTAGCCGAGACACCAC.

Data Analysis

ITS1 sequences isolated from *C. carinata* were of three types, denoted A1, A2, and B. These were aligned to published digenetic ITS1 sequences using CLUSTAL W (Thompson et al. 1994) (Table 1), omitting only published digenetic ITS1 sequences either completely identical at their 3′ end to sequences already present in the data set or for which complete 3′ end sequences were not available. The alignment was thereafter manually corrected with reference to known monophyla. Only the different species of the same genus or family were regarded as known monophyletic groups. Further adjustments of the alignment were performed taking into account the presence of conserved secondary structure elements which were inferred for each DNA sequence independently using Zuker’s (1989) algorithms as implemented in the program MFOLD (Zuker 1989).

The information content of the data set was studied via an analysis of apomorphies in accordance with Wägele (1996b) using the program PHYSID (unpublished; program available from J.-W. Wägele). The analysis of apomorphies aims at the identification and assessment of putatively derived character states (putative apomorphies) in a molecular data set. It is based on the assumption that, by virtue of their closer relationship, substitutions are expected to have occurred less frequently within a monophylum than among outgroup taxa or between outgroup taxa and the considered monophylum. Putative apomorphies are therefore found at those alignment positions which show all identical nucleotides for a tested monophylum that differ from those of the corresponding outgroup. Such positions may show (i) a symmetrical character-state distribution (symmetrical split-supporting positions) in which there are identical nucleotides within both the in- and the outgroup, with differences found only between these groups, and (ii) an asymmetrical character-state distribution (asymmetrical split-supporting positions) where only the ingroup bears all identical nucleotides, which again differ from those of the outgroup, which in this case

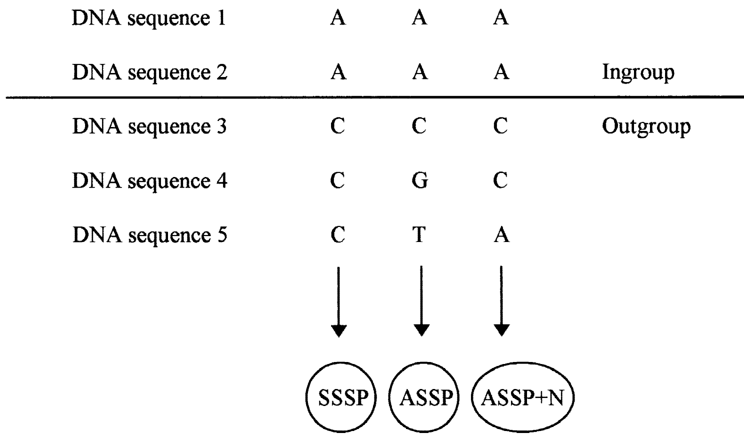

Fig. 1. Example of different character state distributions of split-supporting positions. The *horizontal line* denotes the split between the tested ingroup and the corresponding outgroup. SSSP, symmetrical split-supporting position; ASSP, asymmetrical split-supporting position; ASSP+N, asymmetrical split-supporting position including ''noise'' in the form of one analogy in the outgroup.

also shows variation (Fig. 1). Such asymmetrical split-supporting positions provide an important indication of character state polarity and might therefore serve in identifying a putative monophylum. The analysis of apomorphies included the following steps: (i) for a putative monophylum, all symmetrical, asymmetrical, and also asymmetrical split-supporting positions which showed a defined degree of ''noise'' (in the form of one analogy in the outgroup) were identified; (ii) the same procedure was repeated for the corresponding outgroup; (iii) all identified putative apomorphies for the tested monophylum were added and set against those identified for the corresponding outgroup, resulting in a spectrum of supporting positions for this particular tested ingroup; (iv) a spectrum of supporting positions was inferred for alternative hypotheses of monophyly; and (v) a comparison of such alternative hypotheses was thereafter used to identify the extent and distinctness of the phylogenetic signal produced by the data such that only those groups which are compatible with each other should be well supported.

The aligned sequences were finally subjected to phylogenetic analysis based on distance approaches, maximum likelihood, and maximum parsimony. Genetic distances were calculated using the Jukes–Cantor, Kimura's two-parameter, and Hasegawa–Kishino–Yano model as implemented in the program MEGA (Kumar et al. 1993). Tree reconstruction was thereafter realized using the neighbor-joining method. Maximum-likelihood calculations were performed with the help of the program DNAML of PHYLIP (Felsenstein 1995), using a randomized input order of sequences and a transition:transversion ratio of 1.0, 2.0, and 5.0. Maximum-parsimony trees were reconstructed with the program PAUP (Swofford 1991) via a heuristic search using branch-swapping with nearest-neighbor interchanges. Bootstrapping was applied to maximum-parsimony analysis using the same settings. A 50% majority-rule consensus tree was calculated from 1000 bootstrap replicates.

Results and Discussion

Digenetic ITS1 Sequences Isolated from C. carinata

Three types of digenetic ITS1 sequences were found, types A1, A2, and B. Among the isolated digenetic ITS1 elements, one type A1- and one type B-ITS1 sequence were each isolated in a continuous fragment together with a complete and a partial 18S rRNA gene, respectively. DNA sequences for these 18S rRNA genes indicated that *C. carinata* is parasitized by two species of the Digenea (details on 18S rDNA sequence analysis will be published elsewhere). Type A1- and type B-ITS1 sequences are consequently considered to be of digenetic origin, too. This was also assumed to be valid for the type A2-ITS1 element, which shows a high overall sequence similarity to type A1.

Type A1-ITS1 elements were found in all studied host populations of *C. carinata*. Seven cloned type A1-ITS1 elements isolated from three isopod specimens from the Bay of Wismar were completely sequenced for both strands. The presence of type A1-ITS1 elements was furthermore confirmed for two additional host specimens from the Bay of Wismar, five from the Flemhuder Meer, two from Battenoord, and two from the estuary of the Rio Cavado via direct sequencing of PCR products for one strand only. Type A2-ITS1 sequences were isolated from a single host specimen from Battenoord for which complete double-stranded sequences were obtained for two cloned fragments. Type B-ITS1 elements were almost completely sequenced for both strands for nine cloned fragments isolated from four host specimens from Battenoord. Type A1-ITS1 sequences were 696 to 702 bp long, with differences resulting from length variability at two mononucleotide repeat regions. Type A2-ITS1 elements had a sequence length of 699 bp and those of type B varied in length between 679 and 681 bp with differences again being due to variation in the length of one mononucleotide repeat region. Type A1- and type B-ITS1 elements showed additional variation at 8 and 13 positions, respectively. The two ITS1 sequences isolated for type A2 were completely identical. In each case, variation within both type A1- and type B-ITS1 elements was only present in single cloned fragments. As PCR amplification was performed using a Taq polymerase without proofreading activity, such differences are likely to be the result of PCR errors and were therefore ignored in the following analysis (sequences for each type of ITS1 element were submitted to the Embl databank; accession numbers AJ001831, AJ001832, and AJ001833).

Types A1 and A2 show sequence differences of 4.15%. Type B produces sequence dissimilarity values of

29.83% to type A1 and 31.26% to type A2. Type B-ITS1 sequences are significantly different. In accordance with analysis of 18S rRNA gene sequences, they are considered to refer to a separate species. Although types A1 and A2 sequences are rather similar, they should also belong to different, although closely related species. The illustrated maximum-likelihood tree (Fig. 5), in which branch lengths represent inferred evolutionary distances, shows that types A1 and A2 are as distant from each other as are other closely related species of the Digenea, e.g., *Schistosoma mattheei* and *S. haematobium* or *Echinostoma caproni, E. paraensei,* and *E. trivolvis.* The data consequently suggest that *C. carinata* is parasitized by three digenetic species, one of them with a broad geographic range inhabiting all studied host populations and the remaining two species being detected in only one location.

Homology of Aligned Nucleotide Positions

Phylogenetic analysis using molecular markers is realized by reconstructing the evolution of the studied molecules. With respect to DNA sequence data, the sequence alignment is crucial, as it produces the hypotheses about the origin of particular aligned nucleotide positions and therefore shows responsibility for the identifiable homologies between different sequences. If the alignment is incorrect, then it is most likely that the following analysis will fail to reconstruct the correct evolutionary history of the studied DNA region and therefore to uncover correctly the phylogenetic relationships of the investigated taxa (e.g., Wägele and Stanjek 1995; Wägele 1996a, b; Morrison and Ellis 1997). Due to the arbitrariness of the chosen optimality criteria, computer alignments are not necessarily correct. We therefore adjusted them manually, taking into consideration the presence of known monophyla in the data set (in this case, different species of the same genus or family) and the position of structural elements. Both approaches are expected to improve the alignment. It is more likely that homologous nucleotide positions will be correctly identified within known monophyletic groups. This strategy follows the philosophy of progressive sequence alignment (Feng and Doolittle 1987; Mindell 1991), although, in this case, relying on prior scientific knowledge and not on tree reconstruction algorithms as implemented in the conventional sequence alignment programs. In addition, the same structural or functional elements are likely to be found at the same sequence regions for different, but related organisms and, thus, represent suitable indicators for homology of sequence positions [e.g., comparative reconstruction of rRNA secondary structures of Noller and Woese (1981)].

Digenetic ITS1 elements show a considerable degree of length variability (Table 1), some of which is due to repetitive elements (Luton et al. 1992; Kane and Rollinson 1994; Kane et al. 1996). Nucleotide sequence divergence in digeneans is therefore expected to be high. This is true for 5′ end ITS1 elements where no sequence similarities could be identified. However, about 350 bp of the 3′ end of ITS1 sequences and, additionally, about 60 bp of the 5′ end of the 5.8S rRNA gene could be aligned for the unidentified digeneans isolated from *C. carinata* and the published digenetic sequences belonging to four orders. Of the 427 aligned sites, 355 positions were variable, of which 346 were found within the 3′ end ITS1 region. Despite significant similarities of pairwise compared sequences, the overall variation at the 3′ end of ITS1 is too high to permit absolute certainty as to the homology of the aligned sequence positions. However, secondary structure elements strongly support the inferred alignment. All in all, seven conserved helices could be identified for all digeneans (Figs. 2 and 3). Although secondary structure calculations are limited by the accuracy of the underlying model and algorithms, and should be considered to be speculative, the following need to be emphasized.

(i) All secondary structures were identified independently for the whole range of digenetic taxa. All seven helices are present at the same position and in almost-identical form. The occurrence of such structures at the same position in all digeneans is unlikely to be due to chance.

(ii) It was demonstrated for yeasts that the 3′ end of ITS1 is of particular importance regarding rRNA maturation. It contains four processing sites, at least one conserved recognition site for one of the processing factors and, additionally, a conserved secondary structure motif, which all play a role in ribosome biogenesis. Such functional constraints were shown to produce primary and also secondary structure conservation in yeasts (Henry et al. 1994; Van Nues et al. 1994).

(iii) Studies in other eukaryotes highlight the potential of ITS1 elements, and in particular its 3′ end, to form secondary structures which show at least some conservation across eukaryotes despite extensive divergence in nucleotide sequence (Michot et al. 1983; Gonzalez et al. 1990; Kwon and Ishikawa 1992; Wesson et al. 1992; Paskewitz et al. 1993; Schlötterer et al. 1994; Bakker et al. 1995; Schilthuizen et al. 1995; Coleman and Mai 1997; Fenton et al. 1997).

Eukaryotic data support our finding of 3′ end ITS1 secondary structure conservation in digenetic trematodes, which is likely to be due to functional constraints regarding rRNA maturation as identified for yeasts. Primary sequence similarity in combination with secondary structure conservation is therefore assumed to originate

```
                                        Ia                                              Ib                  IIa               IIa  110
A1   :  CGGGGTGTCTG-CCTGT---CTGAAGCTTCTGGGGTGCTCGCGACTAT-TCA-GTTGCTAGTCCACTTCGG-GGGTGACGGGTTGTGCTGTCGA----GTGAGGCAGTGC
A2   :  ..........-.....---.......C....................-.TG-.................-..........................----..........
B    :  .........A-.....---....T..C..C..T.A.......TC..G...T..C.TC.TG.T..CC...-T.........A.......C.TGTGTG...GA....C..
Dsy  :  .A.....C..T-.T......---....T....GC........T..A.T.C.-----A....C...T..TC.T.C-.........A.A..........TT----C.C........
Dsp  :  .A.....C..C-.TC...---....T....G....A....T..A.TCT.G.---A...TC...T..TC.T.T-.A......A.A..........TT----C.C........
Oba  :  .A.....C..A-..C..---........CT.TC....TG.TAATGGT.T..GAA...T.......TAC.T.-..........C.........TTT-----CG........
Lel  :  .A........A-..C..---....T...CT.AC....TG.TAATAGT.C.TGAA...T.......TGC..CT..........C.........TT------CG.A......
Lra  :  .A........A-..C..---....T...CT.AC...ATG.TAATGGT.C..GAA...T.......TAC..CT..........C...A.....CT------CG........
Sma  :  ......AC..AT..CA.GAT.GT.T..CCTGAT....T..T..TAAC.T..GG.....CT.AT.TGCCAA.-..CGATG..ACA....ATGACGC--TA.TGTTGT....
Sha  :  ......AC..AG.......---.GT.T..CCTGAT....T..T..TAAC.T..GG.....CT.AN.TGCCAA.-..CGATG..ACA....ATGACGC--TA.TGTTGT....
Eho  :  .A........A-..C..---A...TC..CTGAT...ATG.ATTCGGTCT..GGACC.TAT.....AGC.A--..AGA.........A...C..T----..C...T.....
Eca  :  .T.....C..A-..C..---A...TA..CTGAT...ATG.AGTTAGCCT..GG.C.T.CT.....AGC.A--..AGA.....C...A...C..T----.ACT..T.....
Etr  :  .T.....C..A-..C..---A...TA..CTGAT...ATG.AGTTAGCCT..GG.C.T.CT.....AGC.A--..AGA.....C...A...C..T----.ACT..T.....
Epa  :  .T.....C..A-..C..---A...TA..CTGAT...ATG.AGTTTGCCT..GG.C.T.CT.....AGC.A--..AGA.....C...A...C..T----.ACT..T.....
Esp  :  .T.....C..A-..C..---A...TA..CTGAT...ATG.AGTTGGCCT..GG.C.TTCT.....AGC.A--..AGA...A.C...A...C..T----.ACT..T.....

                         IIIa              IIIb                                                          IVa                 220
A1   :  TAGGCTTAAAGAGTGGTGTC-TCGGCTACGGCCAGCCCACCGCCCTGGATGTTTTTATTTCC-AACATTTTAC--ACTGTTCAAGTGGTTCGGATCGGCTTTG--TCGTT
A2   :  ....................-.A...............................G.....-..........--....................A..........--.....
B    :  ...A..C..............TG......C.T..............C.C....G...G...GT-CT........--..G...G.....AA....AGC...-..AC--C..G.
Dsy  :  .........T.........TGTT..A.........T.CG..........TT.--CG..G-..GAACCA........--......A..........AAG.T....C.C.GTCA.C.
Dsp  :  ...................TCG--...........T..G..........TC..ACA..GG..TAA.CG..CCA..CT....................A.G.TA....C.GC.A..C
Oba  :  ...................GT---...C.....TTAT..T.........CT..AA.C.G.....C.T........--.....T..........AT----.....----CG..G
Lel  :  .........T.........CT---A..C.A...TTAT............TC..A--..G.....T.T........--.....T..........AT----.....----CG.C.
Lra  :  .........T.........CT---A..C.A...TTAT............TT..A--..G.....T.T........--.....T..........A-----.....----CG.C.
Sma  :  ....T.C......AAT...A-.GCTA..T.CATGCAAAT......C.TTAT.G..CC.A.TTC..AC......--......G...C.A.C...T.T.....GC-CATTCA
Sha  :  ....T.C......AAT...A-.GCTA..T.CATGCAAAT......C.TTAT.G..CC.A.TTC..AC......--......G...C.A.C...T.T.....GC-CATTCA
Eho  :  ......C......GA.AT.T--G.........T.CTG.TC.........TG.TC.G..CAA.TACT..T-A....--......A.........A.GC.TGT.....G---C.AC.
Eca  :  .........T...GA.AT.T--GA.........TCTG.TC.........T..TC.G..TCG.TACC...-.....--.................GC.TGT.....G---C.AC.
Etr  :  .........T...GA.AT.T--GA.........TCTG.TC.........T..TC.G..TCG.TACC...-.....--.................GC.TGT.....G---C.AC.
Epa  :  .........T...GA.AT.T--GA.........TCTG.TC.........T..TC.G..TCA.TACC...-.....--.................GCTTGT.....G---C.AC.
Esp  :  .........T...G..AT.T--GA.........TCTG..C.........TG.TC.G..TC-.TACTG..-.....--...............A.GC.TGT.....G---C.AC.

         IVb                 Va                 Vb                      VIa            VIb                          330
A1   :  TCGTTCCATTGCCC-CAACATGCACCCGG-TCTTGTA-CTGGACTGCATGT-GCAGTC--GCC-TGGA-GGTGCCTTATCCCGGGCTGGACTGAGAAACTTTATCTCGGG
A2   :  .T............-..............-.......-.............-......--...-....-...........................................
B    :  ...C.T........-.........G.T...-.....AG-....GT.......-......--...-....-.A.....G...T....................A.C.C....
Dsy  :  .G.G.-.........T-........G.T.TT.-CG.-.AG-..T..T........-T.....--...-...C-..............A..TA............----CT...TT
Dsp  :  .G.G.-..........A........G.T.TT.-CG.-.AG-..T..TC.......GT.....--...-...C-......C......A..TA............----CT...TT
Oba  :  .G.A.-......A..-.G..........A.-C..C..G-.........T....-.......--...-...C-...............................TGCT..T.A..CA...T.
Lel  :  .G.A.-......A..-............A.-C..C..G-..............-....GTTCN..-...C-...............................TGCT..T.A..CA...TT
Lra  :  .G.A.-......A..-..............-C..C..G-..............-.......CG...C...CT...............................TGCT..T.A..CA...TT
Sma  :  CG.G.TTGC....T--GG........T..-CT.C..G-..............---.CG.TG..T-.A.C-...AAA-.....TA.....C.GC.TT..C.A...GT..TAT
Sha  :  CG.G.TTNC....T--GG........T..-CT.C..G-..............---.CG.TG..T-.A.C-...AAA-.....TA.N...C.GC.TT..C.A...GT..TAT
Eho  :  CGTAG......A..-TC...........TC.....GG.........C..---.CGT--..N-C..C-.......-..........TA....A.-T..CT.GGTCTCT.AT
Eca  :  CGTAG......A..-TCG........T..TC.....GG.C........C..---.CG.--..N-C..C-.......-.........T...G....T..TT.GGTCTAT---
Etr  :  CGTAG......A..-TCG........T..TC.....GG.C........C..---.CG.--..N-C..C-.......-.........T...G....T..TT.GGTCTAT---
Epa  :  CGTAG......A..-TCG........T..TC.....GG.C........C..---.CG.--..N-C..C-.......-.........T...G....T..TT.GGTCTAT---
Esp  :  CGTAG......A..-TCG........T..TC.....GG.C........C..---.CG.--..N-C..C-.......-.........T...G....T..TT.GGTCTAT---

        VIIa              VIIb           ⌈5.8S                                                          427
A1   :  -TTTTCGGTCATGCGCCGGATTCTCGGGT-TACGTACAA-CTCTGATCGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGCAGCCAACTGTGTG
A2   :  -............................-.........-.............C............................................
B    :  -..A..C..T.AT..G.TT.A...-....C..T.C....-..........................................................
Dsy  :  -CA..T..GA.A---..ACT.GA...A.GT.-.......-......G...................................................
Dsp  :  -CA..T..GA.A---..ATT.GT...A.GT.-.......-......G...................................................
Oba  :  -CAACT..GTGA---.T..T.GTG..N.GTG.-......-......G.......GN..........................................
Lel  :  -CA.CTT.GTGA---.GA...GTG..A.GTG.-......-......G...................................................
Lra  :  -CA..G..GTGA---..T...GTG..A.GTG.-......G......G...................................................
Sma  :  GCA..T..GA.AC.ATT.T..GGGATTA.TGG.......-..T...G............................T......................
Sha  :  GCA..T..GA.AC.AAT.T..GGGATTA.TGG.......-..T...G............................T......................
Eho  :  -.G.CTA.CTCGCTAGATAG.-.GG.ACCTA.T......-......A...................................................
Eca  :  -------------------------.ACCAA........-......G...................................................
Etr  :  -------------------------.ACCAA........-......G...................................................
Epa  :  -------------------------.ACCGA........-......G...................................................
Esp  :  -------------------------.ACCAA........-......G...................................................
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Fig. 2. Alignment of 3′ end ITS1 and partial 5.8S rDNA sequences of digeneans. ‘‘[5.8S,’’ putative start of the 5.8S rRNA gene. I–VII, helices which are conserved across digeneans; see also Fig. 3. Abbreviations of species as in Table 1 and text. This figure was generated with the help of the program GENEDOC (Nicholas and Nicholas 1997).

from identity by descent and not chance. Consequently, the sequence alignment is considered to consist of homologous nucleotide positions and therefore be applicable to phylogenetic analysis.

Consistency in the Phylogenetic Signal

DNA sequences which can show only four character states do not allow a simple distinction between homologous and analogous similarities. The probability of ho-

Fig. 3. Secondary structure model of the 3′ end ITS1 rDNA of A1. I–VII refer to identified helices which are conserved across digeneans. The putative start of the 5.8S rRNA gene is indicated by ''–5.8S.'' The secondary structure model was created using the program RNAVIZ (De Rijk 1997).

mology of particular character states can be estimated in the course of a phylogenetic analysis, but if the sequence data are confounded by a high degree of homoplasy, it will be almost-impossible to identify the relevant phylogenetic signal and the reconstructed tree is likely to be wrong (Wägele 1996a). We tested the suitability of digenetic 3′ end ITS1 sequences via an a priori assessment of the phylogenetic signal using an analysis of apomorphies in accordance with Wägele (1996b). This approach analyzes the general presence and distribution of DNA sequence similarities to identify putatively derived character states (putative apomorphies). The method does not rely on specific models of molecular evolution, as it directly uses the pattern present in the sequence alignment, without any further transformation of the data set. It therefore allows an independent and straightforward assessment of phylogenetic signal consistency via a comparison of all groups which are well supported by putative apomorphies. In the ideal case of an unequivocal phylogenetic signal, only taxon combinations which are compatible with each other should be supported.

The analysis of apomorphies was performed for various arrangements of digenetic taxa as illustrated in Fig. 4. Only a few groups are well supported by putatively apomorphic character state distributions such as symmetrical, asymmetrical, and asymmetrical split-supporting positions including ''noise.'' These are AB, ABD, ABDOL, OL, E, and S. It is obvious that these putative monophyla are compatible with each other, as AB falls within ABD, ABD and OL fall within ABDOL, and E and S are separate groups. The high number of ''noisy'' asymmetric split-supporting positions of ABDL result exclusively from allowed convergencies which are present in O. This is in agreement with high support for the monophyly of ABDOL. In conclusion, the analysis of apomorphies demonstrates overall consistency of the phylogenetic signal, which should represent a suitable basis for phylogenetic tree reconstruction procedures.

Congruence Between Phylogenetic Trees

Phylogenetic tree reconstruction methods such as distance approaches, maximum likelihood, and maximum parsimony all produce identical trees for 3′ end ITS1 and partial 5.8S rDNA sequences of digenetic trematodes. As the exact systematic position of the digenetic order Strigeiformes is uncertain (e.g., Rohde et al. 1993) and therefore it is unknown if the schistosomes or the Echinostomatiformes must be regarded as the outgroup taxon of our data set, all calculations for phylogenetic trees were repeated with either taxon as an outgroup. Such manipulation did not result in any alterations either of the

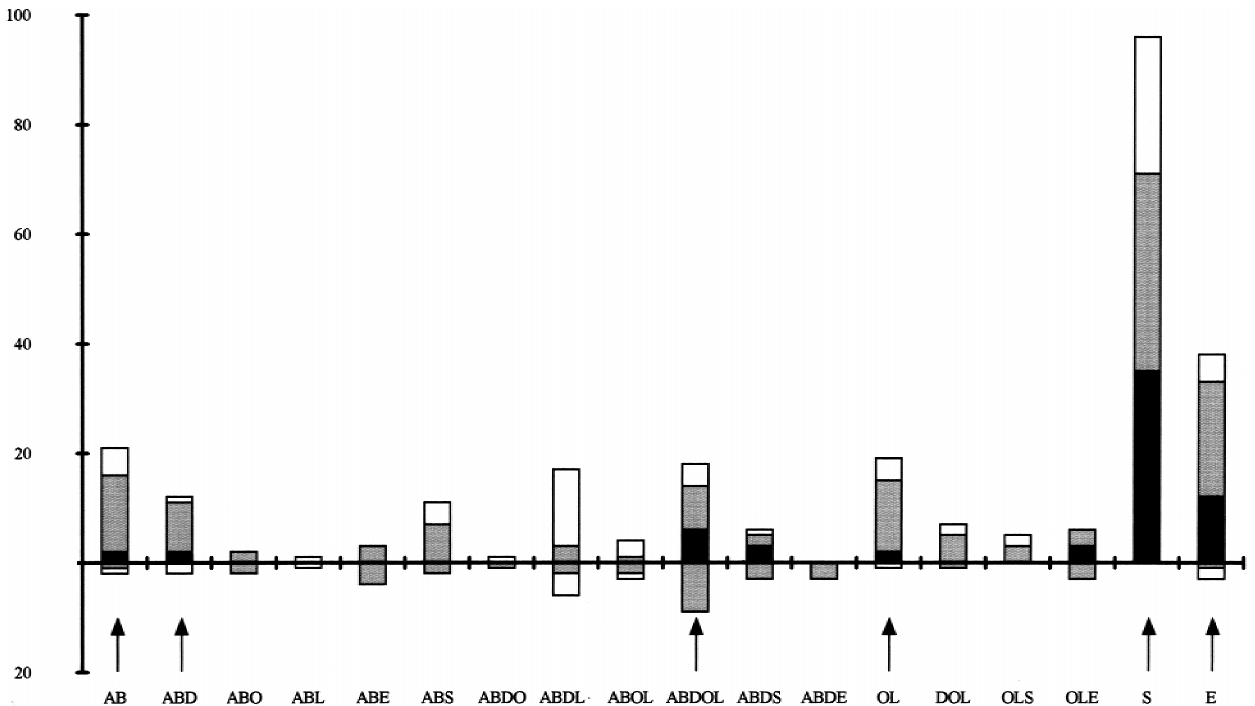

Fig. 4. Analysis of apomorphies for ITS1 sequence data of digeneans. The *X* axis denotes the split between the in- and the outgroup; heights of bars refer to absolute numbers of apomorphies, such that putatively apomorphic characters which are identical between the in- and the outgroup are given only for the ingroup. *Black areas,* symmetrical split-supporting positions; *gray areas,* asymmetrical split-supporting positions; *white areas,* asymmetrical split-supporting positions including ''noise.'' Only those positions were scored as ''noisy'' asymmetrical split-supporting positions which showed no more than one analogy in the outgroup and none in the ingroup. Abbreviations *below columns* refer to tested ingroups. AB—A1, A2, B; D—*Dolichosaccus symmetrus, D.* sp.; O—*Opechona bacillaris;* L, *Lepidapedon elongatum, L. rachion;* S—*Schistosoma mattheei, S. haematobium;* E—*Echinostoma hortense, E. caproni, E. trivolvis, E. paraensei, E.* sp. *Arrows* below the *X* axis indicate well-supported groups.

topology of the tree or of the general statistical support for the resulting clades. Figure 5 depicts a maximum-likelihood tree, and Fig. 6 a maximum-parsimony tree including results of 1000 bootstrap replicates. All clades are supported by high bootstrap values and are also identical to those which were indicated by the analysis of apomorphies. Moreover, there is consistency between our results and phylogenetic trees based on morphological evidence. Although our analysis included taxa of only four trematode orders (Strigeiformes, Echinostomatiformes, Lepocreadiiformes, Plagiorchiformes), morphological studies confirm that, among these, the Plagiorchiformes and Lepocreadiiformes form a monophyletic group (Brooks et al. 1989).

Systematic Position of Unidentified Digeneans Isolated from the Isopod C. carinata

The three genotypes of the unidentified digeneans of *C. carinata* are always clustered in a monophyletic group and fall within the clade consisting of the Plagiorchiformes and the Lepocreadiiformes. Almost all trematodes which are known to parasitize arthropods, including crustaceans, belong to these two orders (e.g. Pearson 1972; Yamaguti 1975; Brooks et al. 1985).

Trematodes have previously also been recorded as parasites of various isopod species (Table 2). The occurrence and distribution of such trematodes are characterized by the following features.

(i) Trematodes recorded as parasites of isopods have all been found to belong to the Plagiorchiata, a suborder of the order Plagiorchiformes. Only one exception has so far been described where the digenean *Allocreadium neotenicum,* a representative of the plagiorchiformean suborder Allocreadiata, parasitizes the isopod *Caecidotea forbesi* (Camp 1992). However, this case is exceptional, as the isopod species serves as the final host, whereas trematodes generally only use arthropods as second intermediate hosts (e.g., Brooks et al. 1985).

(ii) Some of the listed isopod species show an ecology similar to that of *C. carinata*. Particularly the species of the genera *Lekanesphaera* and *Idotea* are also typical inhabitants of brackish water environments and were also recorded for the same locations as *C. carinata* (Amanieu et al. 1979; Olafsson and Persson 1986; Sconfietti 1988; Arrontes and Anadon 1990; Junoy and Viéitez 1992; Franch and Ballesteros 1993).

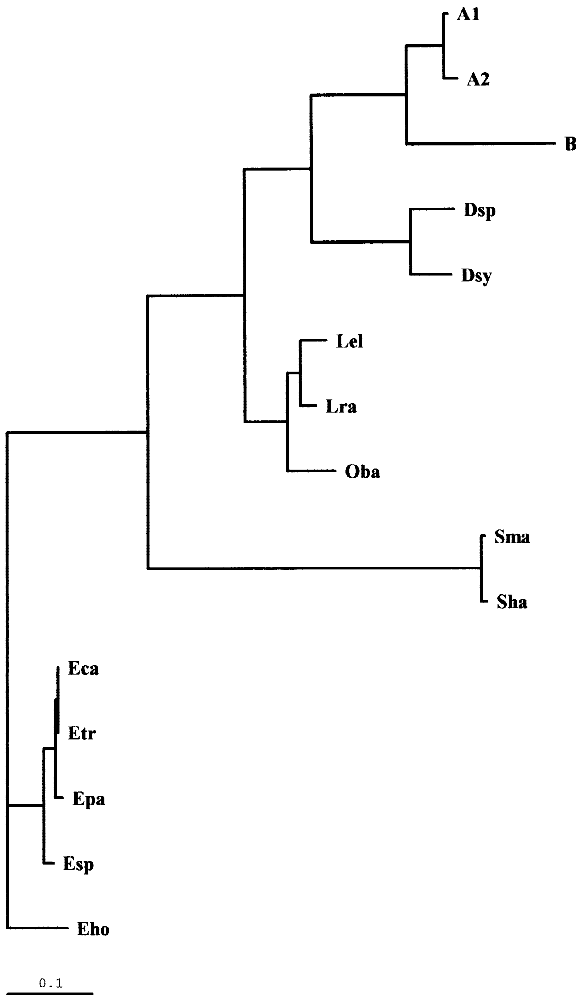

Fig. 5. Maximum-likelihood tree for digenetic ITS1 sequences, generated using DNAML of PHYLIP (Felsenstein 1995) with a transition: transversion ratio of 2.0; randomized input order of sequences which was repeated 10 times; outgroup, *Echinostoma hortense.* An evolutionary distance of 0.1 is given by the *bar* in the *bottom-left corner.* Abbreviations of species as in Table 1 and text.

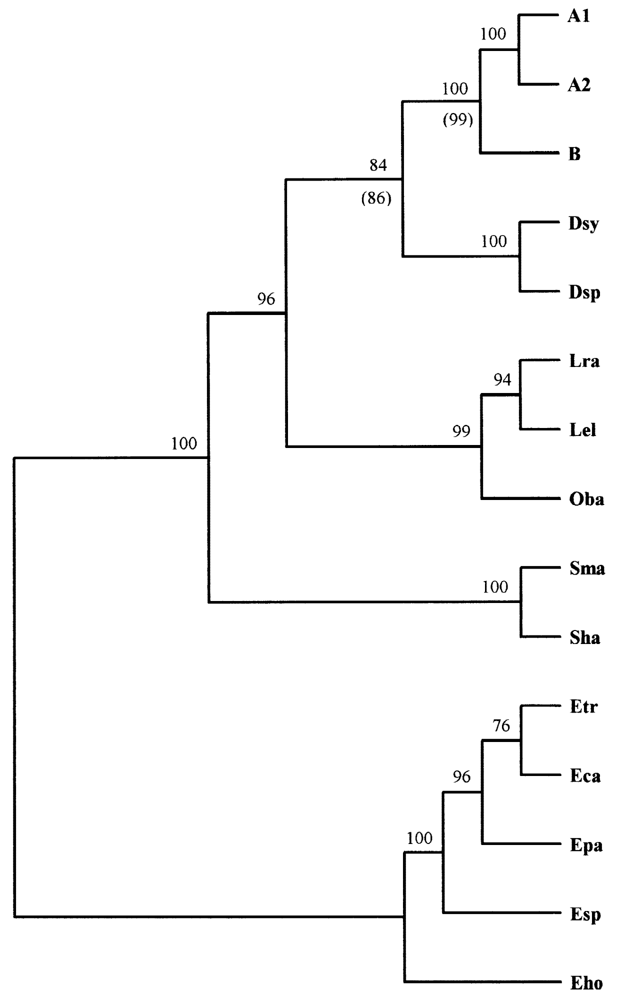

Fig. 6. Bootstrap consensus tree based on maximum parsimony for digenetic ITS1 sequences, generated using PAUP (Swofford 1991) via a heuristic search with branch-swapping, nearest-neighbor interchanges, 1000 bootstrap replicates, and 50% majority-rule consensus tree; outgroup, all species of the genus *Echinostoma.* Bootstrap values are given *above branches. Numbers in parentheses* below branches refer to differences in bootstrap support when the *Schistosoma* species were used as an outgroup. Abbreviations of species as in Table 1 and text.

(iii) Single trematode species use different isopod species as second intermediate hosts. Such a low degree of specificity toward the second intermediate host is even more pronounced than illustrated in Table 2, as digeneans such as *Podocotyle atomon, Microphallus papillorobustum, M. claviformis,* and *Maritrema subdolum* have also been recorded as parasites of various other crustaceans (e.g., Reimer 1970; Køie 1981; Voigt 1991; Bick 1994; Gollasch and Zander 1995; Kesting et al. 1996).

(iv) Single isopod species were found to be host to different trematode species.

In conclusion, *C. carinata* is likely to be parasitized by digeneans of the order Plagiorchiformes, as only these are known to have invaded the host-‘‘habitat’’ Isopoda. *Cyathura carinata* might even be predicted to be host to those listed digeneans of the suborder Plagiorchiata which show a low degree of specificity toward their second intermediate host and also parasitize crustaceans which share the same habitat as *C. carinata*. Phylogenetic analysis of 3′ end ITS1 sequences lends strong support to at least the first hypothesis. Within the clade consisting of the Lepocreadiiformes and the Plagiorchiformes, the unidentified digeneans of *C. carinata* form a monophyletic group with the representatives of the Plagiorchiformes. Since, according to morphological evidence, the clade of the Lepocreadiiformes and the Plagiorchiformes does not contain any further taxon (Brooks et al. 1989), the digeneans isolated from *C. carinata* should belong to the order Plagiorchiformes. As the two representative of the order Plagiorchiformes additionally belong to the suborder Plagiorchiata, the digeneans of *C. carinata* might, furthermore, be members of this suborder too. In addition, the occurrence of trematodes as parasites of isopods also confirms the finding

Table 2. Digeneans as parasites of isopods

Parasite species[a]	Host species[b]	References[c]
Podocotyle atomon (Plagiorchiata: Opecoelidae)	*Idotea balthica* (Valvifera), *I. chelipes*, *I. viridis*, *Lekanesphaera hookeri* (Sphaeromatidea)[d]	1–6
Microphallus papillorobustum (Plagiorchiata: Microphallidae)	*Jaera albifrons* (Janiroidea), *Idotea balthica* (Valvifera), *I. chelipes*, *Lekanesphaera hookeri* (Sphaeromatidea), *L. rugicauda*, *L. serratus*	5–7
Microphallus claviformis (Plagiorchiata: Microphallidae)	*Jaera albifrons* (Janiroidea), *Idotea balthica* (Valvifera), *I. chelipes*, *Lekanesphaera hookeri* (Sphaeromatidea), *L. rugicauda*	5–7
Maritrema subdolum (Plagiorchiata: Microphallidae)	*Jaera albifrons* (Janiroidea), *Idotea balthica* (Valvifera), *I. chelipes*, *Lekanesphaera hookeri* (Sphaeromatidea), *L. rugicauda*	5,6
Maritrema linguilla (Plagiorchiata: Microphallidae)	*Ligia oceanica* (Oniscidea)	8,9
Maritreminoides obstipus (Plagiorchiata: Microphallidae)	*Asellus communis* (Asellota)	7
Megalophallus reamesi (Plagiorchiata: Microphallidae)	*Ligia baudiniana* (Oniscidea)	10
Spelophallus amnicolae (Plagiorchiata: Microphallidae)	*Asellus communis* (Asellota)	7
Allocreadium neotenicum (Allocreadiata: Allocreadiidae)	*Caecidotea forbesi* (Asellota)[e]	11

[a] All listed digeneans belong to the order Plagiorchiformes. General systematic classification of digeneans according to Brooks et al. (1985, 1989).
[b] Isopods serve as second intermediate hosts except where indicated.
[c] 1, Uspenskaya (1963); 2, Reimer (1970); 3, Køie (1981); 4, Zander (1992); 5, Gollasch and Zander (1995); 6, Kesting et al. (1996); 7, Yamaguti (1975); 8, Newell (1986); 9, Benjamin and James (1987); 10, Overstreet and Heard (1995); 11, Camp (1992).
[d] Renaming of the listed *Lekanesphaera* species (originally *Sphaeroma*) according to Jacobs (1987).
[e] The isopod *Caecidotea forbesi* is used as the final host.

that *C. carinata* is host to three species, as multiple infections of single host species seems to be common.

Utility of Digenean ITS1 Sequences

In contrast to the general notion that this ribosomal spacer is too variable for higher-order phylogenetic analysis, the overall consistency of the results suggests that at the 3′ end more than half of the ITS1 element represents a suitable marker for the inference of phylogenetic relationships across the whole digenetic subclass. In addition, due to its high degree of 5′ end variability, complete ITS1 sequences allow simultaneous characterization of digenetic trematodes on different systematic levels. Whole spacer sequences can be used to identify single species unequivocally, whereas 3′ end ITS1 sequences provide information about their systematic position. This bimodal utility of ITS1 elements might be of particular value for the investigation of digenetic life cycles. Due to the paucity of suitable characters in combination with the sometimes pronounced variability of the few available traits, various stages of digeneans are often difficult to identify [see, e.g., the descriptions of metacercaria of *Podocotyle atomon* given by Reimer (1970) and Køie (1981)]. In addition, for many digeneans, particularly those which show a reduction in host specificity, the whole range of host organisms is expected to be unknown. A molecular genetic analysis using ITS1 elements allows rapid screening of large numbers of putative host organisms, assignment of the various stages of a single species, and characterization of the approximate systematic position of unidentified specimens. ITS1 elements may therefore prove useful in extending our knowledge of the diversity of life-cycle patterns of digenetic trematodes.

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