Evolutionary Relationships of Sibling Tapeworm Species (Cestoda) Parasitizing Teleost Fishes

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DNA/DNA hybridization and sequencing of rDNA (partial 18S rDNA and ITS1) were used to investigate phylogenetic relationships among seven host-specific Bothriocephalus parasites (Cestoda, Pseudophyllidae). The small nucleotide divergence between six of the seven bothriocephalids suggests that isolation and differentiation of Bothriocephalus lineages in the different host species probably occurred recently and over a short time span. Comparison of the molecular phylogeny of the parasite species to the phylogeny of their hosts (teleostean fishes) revealed little congruence between the branching patterns of hosts and parasites, suggesting that bothriocephalids have not co-speciated with their hosts.

Introduction

Tapeworms of the genus Bothriocephalus (Cestoda, Pseudophyllidae) have been described as endoparasites in a large number of teleost fishes belonging to several major lineages of Pleuronectiformes, Scorpaeniformes, Gadiformes, and Anguilliformes (Protasova 1977). The first described species, Bothriocephalus scorpii (Mueller 1776), originally reported from the bull-rout (Myxus scorpius), the brill (Scophthalmus rhombus), and the five-bearded rockling (Ciliata mustela). However, studies carried out on the population genetic structure of this parasite have shown strict reproductive isolation between the parasite populations of each host species (Renaud, Gabrion, and Pasteur 1983, 1986; Renaud and Gabrion 1984). These analyses have revealed that B. scorpii constituted a complex of sibling biological species comprising a number of similar morphological species with host preference. Thus, whether B. scorpii is only parasitizing the bull-rout, B. gregarius is found in the turbot, B. barbatus is found in the brill, and B. funiculus is found in the five-bearded rockling. In addition, it is known that two other pleuronectiform fishes, the scaldfish (Arnoglossus laterna) and the spotted flounder (Citharus linguatula), and one anguilliform, the eel (Anguilla anguilla), harbor three other Bothriocephalus species, B. clavibothrium (Ariola 1899), B. andresi (Porta 1911), and B. claviceps (Goeze 1872), respectively.

Thus, the high degree of biological specificity of the different parasite species among hosts belonging to so distantly related orders of teleost fishes (Lauder and Liem 1983) raises the question concerning the compared host–parasite phylogenesis in this heterospecific assemblage. We might ask if concomitant speciations (co-speciation) have occurred, leading to an evolutionary picture where the parasites’ phylogeny mirrors the hosts’ phylogeny (Fahrenholz 1913). Several analyses of co-evolutionary processes have been conducted (Brooks and Glen 1982; Mitter and Brooks 1983; Bandoni and Brooks 1987; Dects 1987; Klassen and Bcvrclcy-Burton 1987; O’Connor 1988; Brooks and McNllnn 1991), all of which involve the appraisal of branching similarities between host and parasite phylogenies and between their taxonomic boundaries or geographic distributions. However, relatively few studies have compared genetic divergence and speciation events in hosts and their parasites, permitting a discussion of the relative timing of speciation within and between host and parasite lineages (Baverstock, Adams, and Beveridge 1985; Hafner and Nadler 1988, 1990; Rannala 1992; Hafner et al. 1994). In the literature, the clearest evidence for co-speciation derives from isozyme electrophoretic data and from sequences of the mitochondrial cytochrome oxidase I gene in a pocket gopher and chewing louse assemblage (Hafner and Nadler 1988; Hafner et al. 1994). These data evidenced synchronous speciation events between sets of coevolving taxa.

The aim of this paper is to estimate the evolutionary relationships of seven species of teleostean parasites (Bothriocephalus) by using two complementary approaches. First, we will employ the nonrepeated DNA/DNA hybridization methodology which allows an estimation of the overall nucleotide divergence between the entire genomes of compared species (Werman, Springer, and Britten 1990). This is the first time that this approach has been applied to parasitic helminths to directly ascertain levels of interspecific genomic divergence. Second, we will use sequence data from nuclear rDNA (the first internal transcribed spacer, or ITS1, and partial 18S rDNA) to investigate the evolutionary relationships within bothriocephalids. Finally, we compare the phylogeny of these parasites with the evolutionary relationships of their teleostean hosts to elucidate biological and ecological factors that may have governed the evolution of this host–parasite association.

Materials and Methods
Specimens Examined

Pleuronectiform fishes were trawled in the Mediterranean Sea, along French coasts (Sete) for turbots, brills, and scaldfishes, and along Spanish coasts (Valen-
cacia) for spotted flounders; scorpaeniforms (bull-rout) and gadiforms (five-bearded rockling) were obtained from the British Channel (Roscoff), and anguilliforms were caught by electrofishing in a river in southern France (Montpellier). After capture, fishes were anesthetized in the laboratory with 3-aminobenzoic acid ethyl ester (Sigma A-5040). Digestive tracts were dissected to collect tapeworms, which were rinsed several times in 0.9% NaCl.

Methods

DNA was extracted from 5–20 individuals of each parasite species, following the procedures of Sambrook, Fritsch, and Maniatis (1989) and Verneau, Renaud, and Catzeflis (1991). Purified DNA was sheared by sonication with a Branson 250 Sonifier calibrated to yield an average fragment size of 500 bp or 2,000 bp, respectively. The size of sonicated fragments was confirmed by agarose gel electrophoresis, where DNA was stained with ethidium bromide and compared with the pBR 322-Taq I marker. Two thousand base pairs of DNA was heat denatured (100°C) and incubated at 55°C until all repeated DNA sequences were reassociated (Verneau, Renaud, and Catzeflis 1991). Fractionation of single-copy versus repeated DNA sequences was performed on hydroxyapatite columns (Britten, Graham, and Neufeld 1974). Twenty-five nanograms of the resulting nonrepeated DNA was radiolabeled with 5-125Iodo-2'-deoxy-cytidine 5'-triphosphate (Amersham, code IM.5103) by using random primer extension (Feinberg and Vogelstein 1983, 1984). Nonincorporated nucleotides were removed from radiolabeled DNA on G50 Sephadex columns (Sambrook, Fritsch, and Maniatis 1989). Duration of the enzymatic reaction was calibrated to yield radiolabeled DNA with a size of 300–500 bp, which was confirmed with sizing gels following standard procedures (Hall et al. 1980; Hunt, Hall, and Britten 1981; Caccione, Amato, and Powell 1987). Nonrepeated radiolabeled DNA, used as “tracer” in the DNA/DNA experiments, was hybridized to a thousand-fold excess of homologous DNA by using the polymerase chain reaction (PCR). This was done for the seven species of bothrioccephalids studied and another parasite Trienophorus nodulosus hosted by Esox lucius, which belongs to the pseudoplyllidean family Ancistrocephalidae. Oligonucleotide primers used for the PCR reactions were designed from regions of 18s rDNA and 5.8s rDNA that had occurred. Therefore, partial sequences of the 18s rDNA, a slow-evolving nuclear gene, were obtained. Preliminary results obtained from the 18s rDNA sequences showed few substitutions between the species studied. So we decided to sequence one intron (ITS1) which evolves more rapidly than 18s rDNA.

Partial 18s rDNA (about 650 bases) and the entire ITS1 region (about 570 bases) were amplified from genomic DNA by using the polymerase chain reaction (PCR). This was done for the seven species of bothrioccephalids studied and another parasite Trienophorus nodulosus hosted by Esox lucius, which belongs to the pseudoplyllidean family Ancistrocephalidae. Oligonucleotide primers used for the PCR reactions were designed from regions of 18s rDNA and 5.8s rDNA that are highly conserved in a wide range of organisms: Caenorhabditis elegans (EMBL accession number: X03680), Opisthorchis viverrini (X55357), Drosophila melanogaster (X70692 and X15707), Alligator mississippiensis (M59383 and M36342), and Mus musculus (X56974). The first primer (L7) hybridized in the 18s rDNA (5'-TGATTGCTGTGGTATTTCCGAT-3') and is homologous to the sequence of the nematode C. elegans at positions 1143–1165 (Ellis, Sulston, and Coulson 1986). The second primer (H7) hybridized in the 5.8s rDNA (5'-GCTCGGTCTTTCATCGATATC-3') and is the complementary sequence of the 5.8s rDNA gene of C. elegans at positions 2132–2154 (Ellis, Sulston, and Coulson 1986). PCR products were immediately cloned in the pGEM-T vector (Promega) and transfected in competent bacteria (JM101 strain of Escherichia coli). Recombinant clones were identified by color screening on indicator plates and were sequenced directly by the method of Sanger, Nicklen, and Coulson among experimental runs of the thermal chromatography (Dickerman 1991). The parameter Tm, which represents the median temperature at which 50% of hybrid DNA has been dissociated, was deduced from the melting curves of DNA hybrids and used for the analysis of evolutionary relationships.

The complete matrix of delta-Tm values was subjected to a bootstrapping procedure to assess its robustness (Krajewski and Dickerman 1990). Resampling of distance data was performed 1,000 times, and the resulting asymmetric matrices were adjusted by using the procedure of Fitch and Margoliash (1967) from each pseudorePLICATE matrix. The percentage of occurrence of each node observed in the resulting networks was calculated by the program Consense in the PHYLIP package (Felsenstein 1990). Because the bootstrapping procedure does not generate normally distributed data (the variance is slightly biased; Weir 1990), a random sample of 100 replicate trees was used to calculate the modal value and 95% confidence interval for each branch length. The resulting network was unrooted.

Because the parasites studied are specific to distantly related hosts belonging to four major fish lineages, we expected to find high levels of molecular changes between bothrioccephalid species if cospeciation had occurred. Therefore, partial sequences of the 18s rDNA, a slow-evolving nuclear gene, were obtained. Preliminary results obtained from the 18s rDNA sequences showed few substitutions between the species studied. So we decided to sequence one intron (ITS1) which evolves more rapidly than 18s rDNA.
For sequencing, we used the Reverse and Forward plasmid primers and two other primers designed
for 18S rDNA and ITS 1 regions. Sequences of these oligonucleotides were as follows: 18S-1 (5'-A'ITGA-
CAATCATGATGGGGCGTAG-3'). For sequencing, we used the Reverse and For-
cwartic plasmid primers and two other primers designed
for 18s rDNA and ITS 1 regions. Sequences of these
oligonucleotides were as follows: 18S- 1 (5'-A'ITGA-
CAATCATGATGGGGCGTAG-3').

Sequences from ITS1 and partial 18S rDNA were
aligned using the MUST package (Philippe 1993). When
alignments were ambiguous, in particular for regions
with insertions or deletions, all sites including a gap
(insertion, deletion) were removed from the phyloge-
etic analyses which were conducted using combined
sequences including all bothriocephalids, T. teocephalidea
bothriocephalid parasites as a monophyletic group (data
not shown). Sequences have been deposited in EMBL
Data Bank under accession numbers YO9670-YO9685.

Results
DNA/DNA Hybridization

Table 1 depicts divergence estimates (average
delta-Tm values with standard deviation) between each
pair of parasite taxa. It was not possible to measure
delta-Tm values between B. claviceps and any other spe-
cies because the level of genomic divergence between
this taxon and the others exceeds the resolving power
of the methodology. The tree (unrooted) is represented
in figure 1. A single branching pattern was observed
between the parasite species where all resulting nodes
are supported at the 100% level.

Nucleotide Sequences

The alignments of 18SrDNA and ITS1 sequences
for the seven bothriocephalid species and T. nodulosus
have been deposited in EMBL under accession numbers
DS28072 and DS28073. The length of the 18SrDNA
region was 647 bp for B. claviceps and 644 bp for all
other bothriocephalids and T. nodulosus. When align-
ments were obtained and compared to the sequence of
C. elegans, bothriocephalids and T. nodulosus revealed
an insertion of about 150 bp within the 18S rDNA. The
first internal transcribed spacer was sequenced com-
pletely for all parasites studied. Length of this intron

![Diagram](image-url)
was similar in all bothriocephalids (from 570 to 574 bp) with *T. nodulosus* having a 546-bp fragment.

The parsimony analysis is based on 61 informative characters, and the strict consensus tree resulting from five equally parsimonious trees (356 steps; consistency index [CI] = 0.97) is shown in figure 2. The Tamura-Nei distances were computed from 307 variable sites on the 1,152 conserved sites and are summarized in table 1. The tree resulting from the neighbor-joining procedure on the pairwise distances is shown in figure 3A. Both analyses revealed an early divergence of *B. claviceps* (parasite of the eel) relative to the remaining bothriocephalids. The analyses also revealed a close relationship between *B. clavibothrium* and *B. andresi*. Branching patterns among the other taxa remain poorly resolved. Although the distance analysis (fig. 3A) shows that the clade composed of *B. barbatus*, *B. gregarius*, *B. funiculus*, and *B. scorpii* may be monophyletic (bootstrap value around 80%), no other cluster is supported at a bootstrap level higher than 70% (figs. 2 and 3A).

**Discussion**

**Evolutionary Relationships Within Bothriocephalid Parasites**

Genetic distances estimated from DNA/DNA hybridization experiments revealed low levels of divergence among most bothriocephalid species (table 1 and fig. 1). The single exception was *B. claviceps*, with delta-Tm distances higher than 15°C when compared with the six other species (data not shown). Phylogenetic trees obtained from both analyses of the sequence data (i.e., parsimony and distance analyses, figs. 2 and 3A) were generally congruent. In both trees *B. claviceps* was widely divergent from all remaining bothriocephalids studied. This latter group was clearly monophyletic (100% bootstrap percentage in both trees). Thus, all these findings demonstrate that speciation of the *B. claviceps* lineage predated speciation of the other bothriocephalid species and experienced a faster rate of DNA evolution (fig. 3A). Furthermore, these data appear to support a recent origin for the six remaining parasites, which are found to be closely related (table 1 and figs. 1, 2, and 3A). Delta-Tm distances of ca. 3.0°C have now been observed between closely related genera, such as within the hominid primates (Sibley, Comstock, and Ahlquist 1990; Caccone and Powell 1989), whose radiation timing is probably not much older than 7–10 Myr. For example, Caccone and Powell (1989) report a delta-Tm of 1.60°C between *Homo* and *Pan* and a delta-Tm of 2.50 between *Homo* and *Gorilla* and between *Pan* and *Gorilla*. For organisms characterized by
a higher rate of nonrepeated DNA change, such as mice and rats, delta-Tm values of ca. 3.0°C correspond to a divergence time of ca. 2–3 Myr (Catzeflis et al. 1993).

**Comparison of Host and Parasite Phylogenies**

Comparison of the branching patterns among hosts and their associated parasites can provide information on cospeciation and host-switching events that have occurred during the evolutionary history of the association. Few molecular studies have been conducted to discern relationships between orders of teleost fishes. Le, Perasso, and Billard (1989) and Le, Lecointre, and Perasso (1993) showed, from 28S rRNA sequence analysis, that pleurocorticiforms (in our survey, four species are examined: P. maxima, S. rhombus, A. laterna, and C. linguatula) are more closely related to scorporiniforms (for example M. scorpius) than they are to gadiforms (for example, C. mustelae) and, finally, to anguiliforms (for example, A. anguilla) which are the most divergent. However, relationships between the major orders examined in this study are moderately supported with low bootstrap values (Le, Lecointre, and Perasso 1993). Concerning relationships within pleurocorticiform fishes, Verneau et al. (1994) showed from DNA/DNA hybridization and electrophoretic analyses that the two scophthalmids (i.e., P. maxima and S. rhombus) are more closely related to each other than either is to a citharid (i.e., C. linguatula) or a bothid (i.e., A. laterna). Moreover, the two latter families (Citharidae and Bothidae) are as divergent from Scophthalmidae as they are from each other (Verneau et al. 1994). So, it seems that speciation events leading to the lineages of C. linguatula and A. laterna occurred before the split between P. maxima and S. rhombus. In the light of a tree based on the combination of these sets of data (Le, Perasso, and Billard 1989; Le, Lecointre, and Perasso 1993; Verneau et al. 1994), we can discuss the evolution of this host-parasite association when the combined host tree (fig. 3B) is compared to the parasite phylogeny (fig. 3A).

Besides the early separation of B. claviceps, all remaining bothriocephalid parasites are grouped in just two clades: (1) B. gregarius, B. barbatus, B. fuciculus, and B. scorpion; and (2) B. clavibothrium and D. andresi (fig. 3A). Thus, flatfish parasites (i.e., tapeworms of Pleurocorticiformes labeled F on figs. 1, 2, and 3A) do not appear to be monophyletic. Consequently, the discrepancies observed in the branching patterns between the two biological assemblages (i.e., hosts and parasites) suggest that pleurocorticiform fishes and their associated parasites have not cospeciated. The branch lengths of the parasites trees (figs. 1 and 3A) suggest that the speciation between B. andresi and B. clavibothrium is more recent than the one between B. gregarius and B. barbatus, whereas the branching pattern of the fishes indicates that P. maxima and S. rhombus are more related to each other than either is to C. linguatula and A. laterna (fig. 3B). As bothriocephalids are subdivided into two clades on the phylogenetic tree of figure 3A, the unrooted network (fig. 1) shows that B. andresi and B. clavibothrium are more closely related to each other than is B. gregarius to B. barbatus. This contrasting pattern in evolutionary relationships between hosts and parasites suggests that a host-switching event has occurred during the evolutionary history of C. linguatula and A. laterna and their parasites.

Two different—but nonexclusive—evolutionary processes can be proposed for explaining the results of this study: (1) sequential colonizations of different host species followed by speciation of parasites, as proposed elsewhere in helminth faunas by Hobberg (1986, 1992) and Hobberg and Adams (1992); or (2) a rapid radiation of a ubiquitous ancestral parasite species, leading to isolation and differentiation of Bothrioccephalus lineages in the different host species. As judged from the small amounts of genomic divergence measured between most pairs of tapeworms, these processes have occurred rather recently and over a short time span.

**Acknowledgments**

We thank Julio Herrero and Santiago Mas Coma for their help in collecting bothriocephalid samples from paraylas in Valencia (Spain), Louis Euzet for his help in collecting bothriocephalid samples from parpeilles in Sete (France), and Roland Marin for his help in electrofishing eels in the south of France. We also thank Rodney Honeycutt, Thierry De Meéus, Guillaume Lecointre, and an anonymous referee for their helpful comments and discussion. Laboratory facilities were provided by Service Commun de Systématique de l’Université de Montpellier II, and Olivier Verneau was financially supported by the Ministère de la Recherche et de la Technologie.

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Rodney L. Honeycutt, reviewing editor