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Olivier Verneau · Frédéric Thomas · Anne de Meeüs  
François Catzefflis · François Renaud

## Evidence of two genetic entities in *Bothriocephalus funiculus* (Cestoda) detected by arbitrary-primer polymerase chain reaction random amplified polymorphic DNA fingerprinting

**Abstract** The genetic diversity of two samples of Cestoda (*Bothriocephalus funiculus*, Renaud and Gabrion, 1984) parasitizing two sympatric teleostean species was assessed using random amplified polymorphic DNA (RAPD). A total of 72 *Bothriocephalus* were analyzed individually, and electrophoretic analysis of the amplification products of 65 primers among the 68 tested revealed monomorphic patterns, reflecting the close genetic relatedness within and between the parasites of the two samples. However, 3 primers showed polymorphic patterns at 6 RAPD sites. Analysis of the distribution of these genomic fragments, assuming random mating, showed strong linkage disequilibria (only 8 genetic combinations were observed among the 32 expected). Two genetic entities displaying a high degree of host specificity were evidenced within our two samples of *B. funiculus*. This powerful molecular technique can be used as a diagnostic tool in studies concerning the biodiversity of related genetic entities and could have broad applications in parasitology.

**Key words** Biodiversity · RAPD · Evolution · Parasitism · Teleostean · Cestoda

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### Introduction

Until recently, information provided by the development of molecular methodologies in evolutionary parasitology

and analyzed through population-genetics concepts has revealed an increase in parasite diversity (Curran et al. 1985; Piessens et al. 1987; Rishi and McManus 1987; Unnasch 1987). Indeed, narrow links (i.e., specificity) between hosts and parasites appear more and more frequently, suggesting parallel evolution in these biological pairs. Such a case has been demonstrated in bothriocephalids of teleostean fishes (Renaud et al. 1983, 1986; Renaud and Gabrion 1984; Ortega and Valero 1989). Using multilocus enzyme electrophoresis (MEE), these different authors showed that far from representing a non-specific species displaying a large geographical distribution, the original species *Bothriocephalus scorpii* (Müller 1776) constituted a complex of biological species highly specific for their host species. Moreover, it has been shown that these species present different biological cycles with strong consequences on the epidemiological transmission and population dynamics of these parasites (Robert et al. 1988). However, on the basis of MEE analysis it was impossible to discriminate between individuals of *B. funiculus* parasitizing two related species of teleost fishes belonging to the genus *Onos*, (i.e., *O. mustela* and *O. tricirratus*: Gadidae, Gadiforms). As MEE analyses were conducted on 12 enzymatic loci, the resolving power of the methodology in investigating the genetic divergence between parasite populations was put into question. Indeed, despite constant methodological progress, the use of genomic markers to study the ecology of natural populations was limited by technical considerations (Hadrys et al. 1992).

Recently, DNA polymorphisms have been detected by random amplified polymorphic DNA (RAPD; Welsh and McClelland 1990; Williams et al. 1990). RAPDs are generated by the amplification of genomic DNA with a single primer of arbitrary nucleotide sequence. The polymorphisms generated function as genetic markers that can be used in taxonomy and population biology studies (Demeke et al. 1992; Hadrys et al. 1992). Indeed, RAPDs have been used for the biosystematic analysis of bacteria (Fekete et al. 1992; Brikun et al. 1994), protozoa (Steindel et al. 1993), yeast (Lehmann et al. 1992),

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O. Verneau · F. Thomas · F. Renaud (✉)  
Laboratoire de Parasitologie Comparée (URA 698, CNRS);  
Université Montpellier II, Pl. E. Bataillon,  
34095 Montpellier CEDEX 5, France

A. de Meeüs  
Centre de Recherches de Biochimie Macromoléculaire  
(UPR 9008, CNRS) BP 5051, Route de Mende,  
34033 Montpellier CEDEX, France

F. Catzefflis  
Institut des Sciences de l'Évolution (URA 327, CNRS),  
Université Montpellier II, Pl. E. Bataillon,  
34095 Montpellier CEDEX 5, France

plants (Vierling and Nguyen 1992; Demeke et al. 1992; He et al. 1992), nematodes (Caswell-Chen et al. 1992), insects (Chapco et al. 1992; Hunt and Page 1992), and mammals (Welsh et al. 1991). Concerning parasitic helminths, RAPDs have been used in sexing *Schistosoma* larvae (Gasser et al. 1991) and to identify *Schistosoma* strains and species (Barral et al. 1993; Dias Neto et al. 1993).

In view of these new results, the aim of the present study was to analyze and discuss polymorphisms evidenced by RAPD multilocus probes within and between two samples of the species *B. funiculus* (Renaud and Gaborion 1984) parasitizing two sympatric teleostean species.

## Materials and methods

### Hosts and parasites

Hosts [i.e., *Onos mustela* (L., 1758) and *O. tricirratu*s (Brünnich, 1768)] were captured by hand under rocks at low tide in front of the biological station of Roscoff (Brittany, France). Fishes were killed using the anesthetic 3-aminobenzoic acid ethyl ester (Sigma A5040) and dissected. Parasites (i.e., *Bothriocephalus funiculus*) were removed from the host gut, washed in a saline solution (0.9% NaCl), and individually stored in liquid nitrogen before DNA analysis. A total of 72 adult worms were obtained, 36 from *O. mustela* and 36 from *O. tricirratu*s.

### *Bothriocephalus* genomic DNA preparation

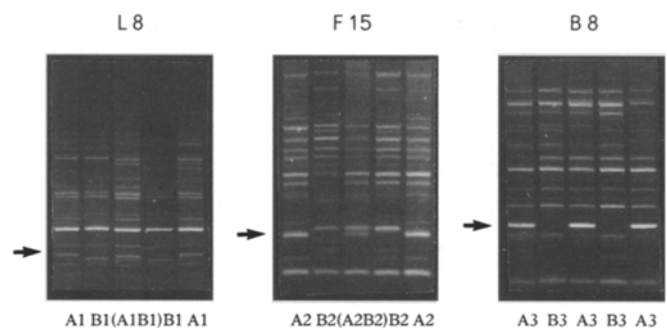
Genomic DNA extraction of Cestoda was individually obtained by phenol extraction and ethanol precipitation according to the protocol defined by Verneau et al. (1991).

### Primers

A total of 68 arbitrary 10-bp primer used for DNA amplifications were synthesized according to the theoretical parameters (i.e., greater than 60% G-C content, no dyad symmetry, and non-self-complementary) and purchased from Operon Technologies Inc. (Alameda, Calif., USA). They were from Operon kits B (15 primers, B2–B16), E (20 primers, E1–E20), F (20 primers, F1–F20), and L (13 primers, L3–L8 and L13–L20).

### PCR amplification

The final protocol-adopted reactions (25 µl) for polymerase chain reaction (PCR) contained 1.0 unit *Taq* DNA polymerase (ATGC), 10 mM TRIS-HCL (pH 8.3), 50 mM KCL, 1.5 mM MgCl<sub>2</sub>, 80 µM of each deoxynucleoside triphosphate (dNTP; Boehringer), 0.5 µM primer (Operon), and 10 ng genomic DNA. This reaction was overlaid with mineral oil and amplification was performed on a thermocycler (Crocodyle II, Appligene) for 1 cycle of 95° C for 5 min (denaturation), 35° C for 1 min (annealing), and 72° C for 2 min (extension), which was followed by 40 cycles of 95° C for 1 min, 35° C for 1 min, and 72° C for 2 min. Reaction products were resolved by electrophoresis at 75 V for 4 h in a 1.5% agarose gel containing TBE 1× and were visualized with ethidium bromide fluorescence. The size of amplified fragments was estimated by comparison with various standard DNA-marker sets (bacteriophage λ double-digested by *EcoRI-HindIII*). Controls were routinely used with no genomic DNA. No amplification occurred in these controls, thus eliminating possible artefactual products and contamination. We also included a control for host contamination,



**Fig. 1** Polymorphic patterns obtained from *Bothriocephalus funiculus* DNA at three RAPD primers (*L8*, *F15*, *B8*). Three different patterns can be observed for the *L8* primer: the first is represented by amplification fragment A1 at 500 pb; the second, by fragment B1 at 460 pb; and the third, by fragments A1 and B1 at 500 and 460 pb, respectively, which represent a combination of the two others. In the same way, we can distinguish three different patterns for the *F15* primer: the first is characterized by fragment A2 at 320 pb; the second, by fragment B2 at 380 pb; and the third, by fragments A2 and B2 at 320 and 380 pb, respectively. The polymorphism observed for the *B8* primer reveals only two patterns, one of which is represented by fragment A3 at 280 pb, whereas the other, named B3, revealed no amplification product. Arrows point to shared bands (A1, B1, A2, B2, A3, B3) referred to above

which always shows amplification patterns quite different from those obtained with parasites. Moreover, to test the quality and the reproducibility of amplification reactions, we repeated each reaction at least once. Markers were scored as being present or absent, and only amplified markers present in a reproducible way were taken into account.

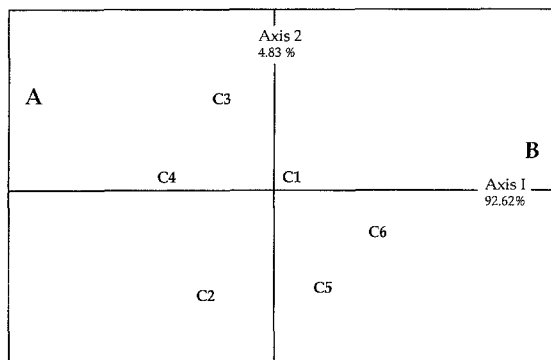
## Results

For the 72 *Bothriocephalus* individuals analyzed, 65 monomorphic amplification patterns were obtained among the 68 primers tested. Amplified product sizes ranged from 0.2 to 1.6 kb. However, the 3 remaining primers *L8*, *F15*, and *B8* revealed polymorphic patterns. The primer sequences were as follows: *L8* (5'-AG-CAGGTGGA-3'), *F15* (5'-CCAGTACTCC-3'), and *B8* (5'-GTCCACACGG-3'). According to the different patterns observed at each of these 3 primers (Fig. 1), the analyzed *Bothriocephalus* can be divided into 8 classes (Table 1). In all, 60 (83%) *Bothriocephalus* can be distributed into 2 genetic groups: 30 are included into type A (fragments A1, A2, and A3 for the three respective primers; Fig. 1) and 30, into type B (fragments B1, B2 and B3 for the three respective primers; Fig. 1). Most type A flatworms (28 among 30) were collected in *Onos mustela*, whereas the majority of type B (29 among 30) were collected in *O. tricirratu*s. The last 12 (27%) *Bothriocephalus* represent combinations of types A and B (Table 1). These results were confirmed by a factorial correspondence analysis (FCA; Benzecri 1973; Lebart et al. 1984), whereby each individual is characterized for each existing fragment (i.e., A1, A2, A3, B1, B2, B3) present at the considered primer. Individuals are then analyzed as active variables using FCA. Thus, this method

**Table 1** RAPD types observed at 3 primers for the 72 *Bothriocephalus* analyzed

Number of <i>Bothriocephalus</i> <sup>a</sup>	Primer L8 types	Primer F15 types	Primer B8 types
<b>(A)</b> 26 M and 4 T	A1	A2	A3
<b>(B)</b> 1 M and 29 T	B1	B2	B3
<b>(C1)</b> 1 M and 2 T	A1, B1	A2, B2	A3
<b>(C2)</b> 5M	A1	A2	B3
<b>(C3)</b> 1M	A1, B1	A2	A3
<b>(C4)</b> 1M	A1	A2, B2	A3
<b>(C5)</b> 1M	A1	B2	B3
<b>(C6)</b> 1T	B1	A2, B2	B3

<sup>a</sup> Letters M and T characterize host species of *Bothriocephalus* (M for *Onos mustela*, T for *O. tricirratu*s). Boldface letters in parentheses correspond to the different groups observed after the FCA analysis (Fig. 2)



**Fig. 2** Variability of different cestodes as studied using FCA for the first two axes. Axes I and II represent 92.62% and 4.83% of the overall respective variability. A, B, and C correspond to the genetic groups defined in Table 1

of analysis characterizes each sample simultaneously according to all the genetic variables (amplified fragments) and shows the contribution of each fragment to the overall variability of the samples.

Figure 2 shows the projection on the plane defined by the two major axes (axes I and II), which represent 97.45% of the overall variability of the samples. Axis I, which represents 92.62% of the overall variability, clearly opposes type A against type B. The other individuals (C1–C6, Table 1) that present combined patterns are distributed between the two groups (A and B).

## Discussion

The large amount of monomorphic amplification products observed for each primer reveals the very close genetic relationships that link these *Bothriocephalus*. However, at 6 independent sites of RAPD primers, this study clearly shows the existence of 2 related genetic entities (i.e., types A and B; 30 individuals for each type among the 72 analyzed) displaying a narrow host specificity.

A question remains concerning the 12 *Bothriocephalus* that present different combination patterns between

the A and B types. Because RAPD fingerprints act as dominant genetic markers (Hadrys et al. 1992), heterozygosity is normally not detectable and allelic frequencies might be difficult to estimate in diploid organisms such as *Bothriocephalus*. However, since *Bothriocephalus* species seem to present random mating (Renaud et al. 1983), it is clear that strong linkage disequilibria occur between the A and B entities. Indeed, we noticed that a large portion of the possible combinations were not encountered during the present study (i.e., only 8 genetic combinations were observed among the 32 expected; Table 1), reflecting a nonrandom mating process between the 2 genetic entities. Two hypotheses can be proposed to explain such a situation: (1) there is no gene flow between the two entities, and the patterns observed for the 12 *Bothriocephalus* could be the reflect of ancestral polymorphisms present in the 2 entities; and (2) a limited gene flow between the 2 entities does occur and, thus, the 3 *Bothriocephalus* presenting the mixed patterns [type C2, Table 1: fragments (A1, B1), (A2, B2), and A3, respectively] could be considered as F1 and the 9 others, as recombinant patterns. It is worth emphasizing that the two host species *O. mustela* and *O. tricirratu*s live in sympatry and, eventually, in syntopy, as they are currently found together under the same rocks (shelter) at low tide. Thus, there is no geographical barrier to a potential gene flow between the two populations of *Bothriocephalus* parasites.

Although RAPD markers can formally be treated as Mendelian alleles (Arnold et al. 1991; Carlson et al. 1991), we cannot settle our question because the genetic determinism of RAPD markers is difficult to elucidate without experimental crosses between the two types A and B. However, our work as well as that of several other investigators (Hu and Quiros 1991; Hadrys et al. 1992) shows that if the RAPD amplification is repeated two or more times, the majority of markers are clearly reproducible and scorable. Thus, this study undoubtedly reveals the existence of two genetic entities preferentially parasitizing one or the other of the two teleostean species. Furthermore, the close genetic relatedness observed between these two entities is in accordance with a previous analysis conducted by one of us (F. R.). Indeed, an electrophoretics study realized on 12 enzymatic loci was not capable of revealing any genetic difference within or between *Bothriocephalus* from the two teleosteans (i.e., *O. mustela* and *O. tricirratu*s). Such a case has been encountered in plants (Hu and Quiros 1991; Martin et al. 1991) and in *Schistosoma* (Barral et al. 1993; Dias Neto et al. 1993), where allozyme studies have not proved to be sensitive enough. Thus, RAPD markers detected by PCR amplification of small inverted repeats scattered through the genome seem to be a new molecular tool in parasitology to investigate the biodiversity and biosystematics of closely related biological entities, whereas morphoanatomical and/or traditional genetics studies are not informative enough. Moreover, this methodology, which necessitates small quantities of DNA, can thus be very useful for the analysis of parasite larvae (e.g., cora-

cidia, plerocercoids, and cercariae, among others) in an epidemiological context.

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