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Research note

Genetic markers in ribosomal DNA for the identification of members of the genus *Anisakis* (Nematoda: Ascaridoidea) defined by polymerase chain reaction-based restriction fragment length polymorphism

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Abstract

Polymerase chain reaction-based restriction fragment length polymorphism analysis was performed to establish genetic markers in rDNA, for the identification of the three sibling species of the *Anisakis simplex* complex and morphologically differentiated *Anisakis* species, i.e. *Anisakis physeteris, Anisakis schupakovi, Anisakis typica* and *Anisakis ziphidarum.* Different restriction patterns were found between *A. simplex* sensu stricto and *Anisakis pegreffii* with two of the restriction endonucleases used *(Hin*fI and *Taq*I*),* between *A. simplex* sensu stricto and *A. simplex* C with one endonuclease *(Hha*I*),* and between *A. simplex* C and *Anisakis pegreffii* with three endonucleases *(Hha*I*, Hin*fI and *Taq*I*),* while no variation in patterns was detected among individuals within each species. The species *A. physeteris, A. schupakovi, A. typica* and *A. ziphidarum* were found to be different from each other and different from the three sibling species of the *A. simplex* complex by distinct fragments using 10-12 of the endonucleases tested. The polymorphisms obtained by restriction fragment length polymorphisms have provided a new set of genetic markers for the accurate identification of sibling species and morphospecies.

Keywords: Anisakis; Genetic markers; Polymerase chain reaction-restriction fragment length polymorphism; Ribosomal DNA; Species identification

In recent years, the taxonomy of anisakid nematodes has been redefined based on multilocus enzyme electrophoretic analyses. These analyses have shown that many anisakid morphospecies (i.e. *Anisakis simplex, Pseudoterranova decipiens, Contracaecum osculatum* and *Contracaecum rudolphii),* previously considered cosmopolitan and able to colonise a broad range of hosts, actually comprise a number of biological species which are reproductively isolated with distinct ecological niches (i.e. represent sibling species) [1-7]. For instance, the morphospecies *A. simplex* consists of three sibling species, namely *A. simplex* sensu stricto, *Anisakis pegreffii* and *A. simplex* C, differing in their genetic structure, life-history and geographic distribution [4]. *Anisakis simplex* sensu stricto is widespread between 30°N and the Arctic polar circle, and *A. pegreffii* is widely distributed in the Austral region as well as in the Mediterranean Sea, *A. simplex* C has

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been found in the Canadian Pacific and in regions south of 35°S [4]. Multilocus enzyme electrophoretic analyses have also allowed the genetic structure of *Anisakis physeteris* to be studied, thus providing genetic markers for the identification of larval forms belonging to this species [3,8]. In addition these have enabled a new species, *Anisakis ziphidarum,* to be detected [9].

The development of molecular markers for the accurate identification of related species can be also achieved by using PCR-based approaches that have shown a remarkable sensitivity in the detection of genetic variation requiring only small amounts of fresh or ethanol-fixed parasite material for analysis. For example, PCR-based restriction fragment length polymorphism (PCR-RFLP) analysis of the ribosomal DNA (rDNA) internal transcribed spacers (ITS-1 and ITS-2) provides a useful approach for the specific identification of both distantly and closely related ascaridoid species, as these spacers showed high levels of interspecific sequence differences in the presence of low-level intraspecific variation [10].

The aim of the present work was to exploit PCR-RFLP to establish genetic markers in rDNA for the identification (irrespective of developmental stages) of the three sibling species of the *A. simplex* complex and another four morphologically differentiated species of *Anisakis,* i.e. *A. physeteris,* a parasite of sperm whales, *Anisakis typica,* a parasite of cetaceans in warm waters, *A. ziphidarum,* a parasite of cetaceans of the family Ziphiidae and *Anisakis schupakovi,* a parasite of the relict phocid species of the Caspian Sea, *Phoca caspica.* The latter species was considered as *inquirendae* by Davey [11] in his revision of the genus *Anisakis,* but retained as a valid species by Kurochkin [12].

Parasite species, number of specimens tested, geographical origins, life-cycle stages and hosts are listed in Table 1. For the three sibling species of the *A. simplex* complex, the study was conducted on specimens previously identified to the species level by multilocus enzyme electrophoresis.

DNA extraction was performed, with minor modifications, according to the protocol proposed by Holmes and Bonner [13]. Each worm was placed in a 1.5 ml Eppendorf tube and kept in liquid nitrogen for few seconds to facilitate the rupture of cell membranes. The tissue was crushed by a pestle in 200 μl Holmes-Bonner buffer (urea 7 M, Tris-HCl 100 mM pH 8.0, EDTA 10 mM pH 8.0, NaCl 350 mM, SDS 2%). Subsequently, the DNA was purified with one phenol-chloroform extraction, followed by two chloroform extractions and then an ethanol precipitation. The precipitated pellet was resuspended in 100 μl TE containing RNase. Two conserved primers A (forward): GTCGAATTCGTAGGTGAACCTGCGGAAGGATCA and B: GCCGGATCCGAATCCTGGTTAGTTTCTTTTCCT [14] were used in PCR to amplify an rDNA region (between the 3' end of the ssrRNA and the 5' end of the lsrRNA genes) using a standard buffer (Perkin-Elmer) under the following conditions: 10 min at 95°C, then 30 cycles of 30 s at 95°C, 30 s at 55°C and 75 s at 72°C, followed by a final elongation of 7 min at 72°C. Amplicons were subjected to PCR-RFLP analysis using 16 individual restriction enzymes *(Alu*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hha*I, *Hin*fI, *Hpa*II, *Hsp*92II, *Mbo*I, *Nci*I, *Pst*I, *Pvu*II, *Rsa*I, *Sac*I and *Taq*I*)* for all species under study.

The amplification of the rDNA region (spanning the ITS-1, ITS-2 and the 5.8S subunit) produced a fragment of ~1 kb. Of the 16 restriction enzymes tested, 12 cleaved the target sequence in one or more of the species under study, while four enzymes *(Dra*I, *Eco*RV, *Pst*I and *Sac*I*)* did not. Different restriction patterns were produced between *A. simplex* sensu stricto and *A. pegreffii* using *Hin*fIand *Taq*I, between *A. simplex* C and *A. pegreffii* using *Hha*I, *Hin*fI and *Taq*I and between *A. simplex* sensu stricto and *A. simplex* C using *Hha*I (Fig. 1), while no variation in RFLP patterns was observed among individuals within each of

Table 1
The species of the genus *Anisakis* under study, with number of specimens tested (No.), geographic origin, life-cycle stage and hosts

Parasite Species	No.	Geographic origin	Life stage[a]	Host species
Anisakis simplex s.s.	8	Pacific Canadian waters	A	*Pseudorca crassidens*
	5	Norwegian waters	A	*Globicephala melaena*
Anisakis pegreffii	5	Tyrrhenian Sea	A[b]	*Micromesistius poutassou*
	3	Tyrrhenian Sea	L3	*Micromesistius poutassou*
Anisakis simplex C	2	Pacific Canadian waters	A	*Pseudorca crassidens*
Anisakis physeteris	2	Tyrrhenian Sea	A	*Physeter catodon*
Anisakis schupakovi	5	Caspian Sea	A	*Phoca caspica*
Anisakis typica	1	Brazilian waters	A	*Stenella longirostris*
Anisakis ziphidarum	2	South African waters	A	*Ziphius cavirostris*

[a]A = adults; L3 = third stage larvae.
[b]Adults of *A. pegreffii* were obtained from in vitro culture.

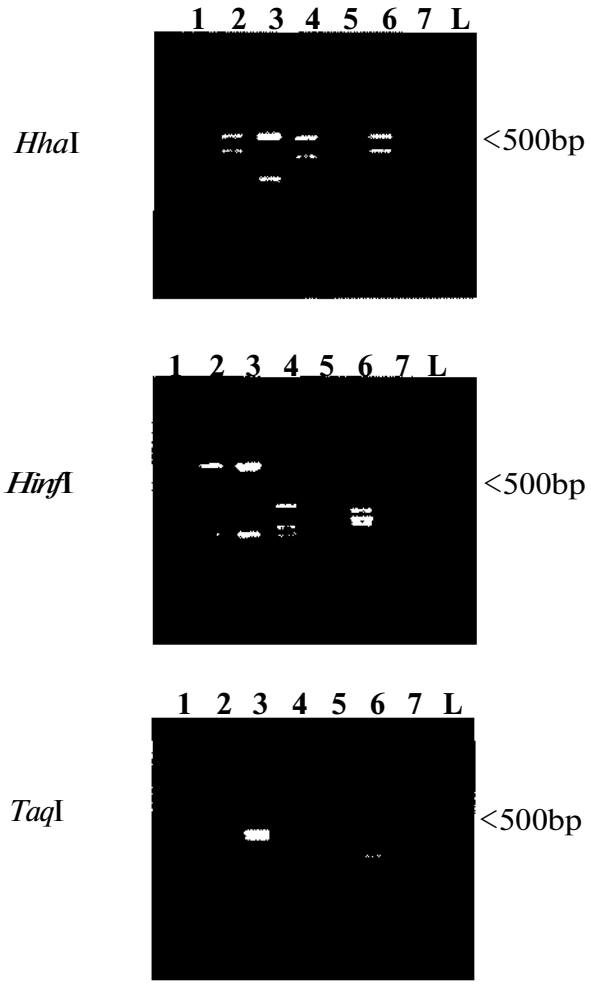

Fig. 1. Restriction fragment length polymorphism patterns of the rDNA region spanning the ITS-1, the 5.8S gene and the ITS-2 shown by the seven species of the genus *Anisakis* under study at three restriction enzymes *(HhaI, Hin*fI,, *TaqI,)*. Lanes: 1, *A. pegreffii;* 2, *A. simplex* sensu stricto; 3, *A. simplex* C; 4, *A. physeteris;* 5, *A. schupakovi;* 6, *A. ziphidarum;* 7, *A. typica;* L, 100 bp ladder.

the taxa. Restriction with *Hhal* produced two fragments of approximately 550 bp and 430 bp in *A. pegreffii* and *A. simplex* sensu stricto, while *A. simplex* C showed the same fragment of 550 bp plus two fragments of approximately 300 and 130 bp, for the presence of an additional restriction site. Restriction with *Hin*fI. produced two fragments of approximately 620 and 250 bp plus a fragment shorter than 100 bp in *A. simplex* sensu stricto and *A. simplex* C, while *A. pegreffii* showed three fragments of 370, 300 and 250 bp. Restriction with *Taql* produced three bands (430, 400 and 100 bp) in *A. simplex* sensu stricto and *A. simplex* C, while *A. pegreffii* showed the same fragment of 400 bp and two fragments of 320 and 150 bp. The results demonstrate that the genetic differentiation among sibling species of the *A. simplex* complex already evidenced by multilocus enzyme electr6phoresis [4] is also detectable in the region spanning the ITS-1, the 5.8S and the ITS-2 of the ribosomal DNA.

Table 2
An example of taxonomic key based on two diagnostic restriction enzymes *(Hha*I and *Hin*fI*)* for the identification of species of the genus *Anisakis*

Enzyme	Fragments	Species
1. *Hin*fI	370-300-250	—• *A..pegreffii*
	380-290-270	—• *A..physeteris*
	520-340-120	—• *A..schupakovi*
	620-350	—• *A. typica*
	370-320-290	—• *A. ziphidarum*
	620-250-80	—•2.
2. *Hha*I	550-430	—• *A. simplex* sensu stricto
	550-300-130	—• *A. simplex* C

The three endonucleases (i.e. *Hha*I, *Hin*fI and *Taq*I*)* also provided diagnostic profiles for the four morphologically differentiated species under study. Digestion with *Hha*I produced clearly different profiles in *A. schupakovi* (400, 300 and two co-migrating fragments of 150 bp) and in *A. typica* (320, 240, 180 and 160 bp). Digestion with *Hin*fI produced different profiles in *A. physeteris* (380, 290 and 270 bp), in *A. schupakovi* (520, 340 and 120 bp), in *A. typica* (620 and 350 bp) and in *A. ziphidarum* (370, 320, and 290 bp). Digestion with *Taql* produced different profiles in *A. physeteris* (300, 280 and 140 bp), in *A. schupakovi* (220, 190, 130 and 100 bp), in *A. typica* (400 and 350 bp) and in *A. ziphidarum* (330, 300 and 140 bp). The difference between the sum of fragment sizes and the amplicons is explained by the presence of some not very visible bands below 100 bp. An example of a taxonomic key based on two diagnostic restriction enzymes *{Hha*I and *Hin*fI*)* for the identification of species of the genus *Anisakis* studied is given in Table 2.

In addition to those three restriction enzymes, at least seven of the nine remaining endonucleases employed were found to differentiate the species *A. physeteris, A. schupakovi, A. typica* and *A. ziphidarum* from each other and from the three sibling species of the *A. simplex* complex, reflecting a higher degree of genetic divergence among morphologically differentiated species than among sibling species. These findings confirm the genetic differentiation of *A. physeteris* and *A. ziphidarum* [3,9] and indicate also that *A. typica* and *A. schupakovi* are well differentiated from the species of the *A. simplex* complex and with respect to the other species of the genus. The genetic differentiation of *A. schupakovi,* resulting in a low percentage of shared fragments, ranging from 3.28% (versus *A. ziphidarum)* to 9.68%, (versus *A. physeteris),* supports the validity of this species, as stated by Kurochkin [12].

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