Structure of Anisakis simplex s.l. populations in a region sympatric for A. pegreffii and A. simplex s.s.
Absence of reproductive isolation between both species

Departamento de Parasitología, Facultad de Farmacia, Universidad de Granada, Campus Universitario de Cartuja, 18071 Granada, Spain

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Abstract

The aim of this study was to determine the genetic structure of A. simplex s.l. populations. This was done by applying PCR-RFLP and RAPD-PCR to 42 specimens morphologically identified as third stage larvae of A. simplex s.l. Of these larvae, 59.26% of those of Mediterranean origin are identified by PCR-RFLP as A. pegreffii. In Atlantic waters, this percentage dropped to 20.00% while A. simplex s.s. represents 66.67%. However, findings seem to suggest that the taxonomic status of both species should be reconsidered owing to: (i) the high gene flow value that we detected between A. simplex s.s. and A. pegreffii; (ii) the short genetic distance between both members of the A. simplex complex; (iii) the fact that hybrid genotypes represent 16.67% of the parasites analyzed and are represented in all the populations studied, except for the Cantabrian one. When the genetic variation is estimated between the 42 individual A. simplex s.l. specimens studied these can be classified into four groups. The genetic distances and gene flow between three of them are compatible with the existence of three different genetic populations. The fourth is comprised of a single specimen of L3, identified both by PCR-RFLP and by RAPD-PCR as a new genotype.

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Keywords: Anisakis simplex complex; Genetic structure of populations; Gene flow; Hybrids genotypes

1. Introduction

Anisakis simplex is the main etiological agent of a human disease called anisakiasis that humans acquires by eating fish subject to little heat treatment or fish that is smoked, soaked in vinegar, pickled with spices and other raw fish specialities. This nematode produces clinical symptomatology characterized by serious gastrointestinal lesions. Moreover, owing to the thermostability of A. simplex allergens, the ingestion of food containing dead parasites can also be potentially dangerous and can cause allergic reactions mediated by IgE that range from potentially lethal anaphylactic reactions of immediate onset to chronic debilitating conditions. A. simplex is becoming increasingly known as an etiological agent in cases of food allergy in the adult population [1].

Over the last few years, isoenzyme electrophoretic studies have shown that the A. simplex morphospecies includes a complex of sibling species [2]. According to these authors, this complex is comprised of at least 3 different species, A. simplex s.s., A. pegreffii and A. simplex C, which have different genetic structures, biological cycles and geographical distributions. These species can also be distinguished by PCR-RFLP analysis of the ribosomal DNA (rDNA) internal transcribed spacers (ITS-1, the 5.8S and the ITS-2) [3].

The coasts of the Iberian Peninsula are geographical areas where the two sibling species, A. simplex s.s. and A. pegreffii, are sympatric and have been found on several occasions parasitizing several fish species captured in different Spanish coastal regions, including blue whiting or Micromesistius poutassou [2,4]. This is a fish commonly eaten in Spain and is the fish species with one of the highest intensities and abundance of anisakid infestation [5,6]. Our objective was to
determine the genetic population structure of *A. simplex* s.l. in specimens of *M. poutassou* captured at different sites on the Spanish Atlantic and Mediterranean coasts, by carrying out a study of the genetic variability and diversity of these parasites and determining gene flow, reproductive isolation and genetic relationships.

2. Material and methods

2.1. Hosts and parasites

We examined a total of 401 blue whiting captured from different sites along the Spanish Mediterranean and Atlantic coasts. After measuring the total length, the fish were dissected and examined to isolate the parasites. The viscera, internal organs and ventral and dorsal musculature were digested separately at 37°C, with a solution of HCl–pepsin, pH 2–2.3. The morphological identification of anisakids is based on a suite of characters as described previously [7–10]. The prevalence of *A. simplex* s.l. infestation was 9.1% in blue whiting captured from Mediterranean sites and 81.7% in those captured on Atlantic coasts. The larvae to be used in the study were directly isolated from the fish and then individually stored at −80°C.

2.2. DNA extraction

Each worm was placed in a 1.5 ml Eppendorf tube and kept in liquid nitrogen for a few seconds to facilitate the rupture of cell membranes. The tissue was crushed by a pestle in lysis buffer (10 μl of SCE (sorbitol 1 M, sodium citrate 0.1 M, EDTA 0.06 M), 30 μl of SDS-EDTA (SDS 1%, EDTA 0.15 M) and 10 μl of proteinase K (1%)) and then incubated at 42°C over night. Subsequently, the DNA was purified with one phenol–chloroform–isoamyl alcohol extraction, followed by one chloroform–isoamyl alcohol extraction and then an ethanol precipitation. The precipitated pellet was resuspended in 50 μl bidistilled water and kept at −20°C until use.

2.3. Identification of sibling species by PCR amplification and restriction fragment length polymorphism (RFLP) profiles

An rDNA region between the 3' end of the small subunit rRNA and the 5' end of the large subunit rRNA was amplified using the primers A (forward): GCTGTGAAATTCAAGGATCTGC and B (reverse): GCCCGGACTGAACCTGCTGCTGATTTCTTCCT following the methodology described by D’Amelio et al. (2000) [3]. Amplicons were subjected to RFLP analysis using three restriction enzymes, Cfo I, Hinf I and Taq I (Boehringer [3]).

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2.4. Random amplified polymorphic DNA (RAPD-DNA)

We performed RAPD-DNA of *A. simplex* s.l. with the following four arbitrary sequence primers: ILO-OO4: GGA-ATTCCGCTTACACTAC; ILO-524: CGCGCCCGC; ILO-868: CAGCCTCGGC and M13: TGACCCGCAGAAAA-TG. Each 25 μl of RAPD reaction contained: 2 mM MgCl2, 0.2 mM dNTPs, 25 pmol primer, 1.25 U Taq polymerase (Bioculter) in the buffer recommended by the manufacturer and 1 μl of DNA. Amplifications were carried out using one cycle of 94°C for 2 min; 40 cycles of 94°C for 30 s, 36°C (60°C for primer M13) for 1 min and 72°C for 2 min; one cycle of 72°C for 2 min. RAPD products were separated on 1.5% agarose gels in TBE buffer, with ethidium bromide, at 90 V for 5 h.

The DNA fragments generated were designated by their molecular weights followed by the name of the primer used for their amplification. Using the RAPD profiles obtained with each primer, a binary matrix was constructed in which each of the parasites studied was represented and all of the characters studied (DNA bands). Each specimen was assigned a value of 1 or 0 for each character according to whether it is present or absent. The characters conserved (DNA fragments present in all the individuals) were given a value of 1 in all of the parasites. The raw data-binary codes for RAPD data are available for any researchers who may want to reanalyse them and can be requested from the authors.

Genetic variability and diversity within and between populations was estimated using the following parameters, respectively: (i) number and proportion of polymorphic loci at the 0.95 criterion; the 0.99 criterion is not appropriate when sample size is smaller than 50 [11], and (ii) Ht (total gene diversity), Hs (diversity within populations) and Gst (coefficient of genetic differentiation). Allele frequencies were estimated assuming: (i) that genomic regions amplified by RAPD-PCR segregate as dominant alleles, (ii) that genotype at RAPD loci are in Hardy-Weinberg proportions in each subset, since this appears to be a normal situation for *Anisakis* species [2,12]. Gene flow was estimated by the Nm parameter from the values of Gst. Genetic divergence of populations was estimated using Nei’s genetic identity and genetic distance. The computer package POPGENE, version 1.31, was used for population genetic analysis and to draw dendrograms. This is a Microsoft Window-based computer package for the analysis of genetic variation among and within natural populations using dominant markers (http://www.ualberta.ca/~yeh/index.htm). Dendrograms based on Nei’s genetic distances were drawn using UPGMA method, and the robustness of each node was tested by bootstrap analysis (100 pseudoreplicates) using PHYLIP version 3.6 (http://evolution.genetics.washington.edu/phylip).
3. Results

We applied the PCR-RFLP and RAPD-PCR techniques to 42 individual specimens identified morphologically as third stage (L3) *Anisakis simplex* s.l. larvae. Twenty-seven larvae were obtained from blue whiting captured in the Mediterranean (Med): 11 from Motril bay (Med-Mo), 7 from the Malaga coast (Med-Ma), and the other 9 from fish captured on the Spanish Levante coast (Med-Le). Of the 15 specimens collected from fish captured on the Spanish Atlantic coasts (Atl), 10 came from blue whiting specimens caught in the Cantabrian sea in northern Spain (Atl-Cant) and 5 from the Atlantic Huelva coast in southeastern Spain (Atl-Hu) (Fig. 1).

3.1. Identification of sibling species by PCR-RFLP

According to the genetic markers defined by D’Amelio et al. (2000) [3] and Abollo et al. (2003) [13], 19 specimens were identified as *A. pegreffii*, 15 corresponded to *A. simplex* s.s. and 7 were hybrids between these two species (Table 1). One Med-Mo specimen was not assigned to any of the previous genotypes (Fig. 2). Six of the recombinant genotypes displayed a hybrid pattern between *A. pegreffii* and *A. simplex* s.s. with both enzymes, Hinf I and Taq I (the genetic markers generated with the Cfo I enzyme were equal in both cases). In contrast, the seventh hybrid specimen showed a Taq I pattern characteristic of *A. pegreffii* and hybrid when the Hinf I enzyme was used.

3.2. RAPD-DNA fingerprinting

Each of the four primers produced a different fingerprint for each parasite, reflecting the individual variability of these helminths. In total, RAPD-PCR generated 143 genetic markers of which 138 (96.50%) were polymorphic.

3.2.1. Genetic variation analysis between *A. pegreffii*, *A. simplex* s.s., recombinant hybrids and the new genotype

We grouped together parasites identified by PCR-RFLP as belonging to *A. pegreffii*, *A. simplex* s.s., recombinant hybrids or the new genotype. From the RAPD-PCR data, we estimated the Nei’s genetic distances between them (Table 2) and used these to construct an UPGMA tree (Fig. 3).

The percentage of polymorphic loci was 80.42% (115) for *A. pegreffii*, 72.03% (103) for *A. simplex* s.s. and 58.74% for the new genotype.

| Taxa of *Anisakis* identified by genetic markers in *M. poutassou* from all the selected localities of both Atlantic and Mediterranean Spanish waters |
|--------------------------------------------------|---------|---------|---------|---------|
|                     Mediterranean Sea                  | Atlantic Ocean |                  | Total   |
|                     Motril bay            | Malaga coast | Levante coast | Huelva coast | Cantabrian coast |
| *A. pegreffii*        | 8          | 2        | 6        | 2       | 1       | 10      |
| *A. simplex* s.s.     | 0          | 4        | 1        | 1       | 9       | 15      |
| Hybrid genotypes      | 2          | 1        | 2        | 2       | 0       | 7       |
| New genotype          | 1          | 0        | 0        | 0       | 0       | 1       |
| Total                 | 11         | 7        | 9        | 5       | 10      | 42      |
Fig. 2. Identification of sibling species by PCR-RFLP profiles. Cfo I: 2 and 3, A. pegreffii; 4 and 5, A. simplex s.s.; 6, new genotype. Hinf I: 2, 5 and 7, A. simplex s.s.; 3 and 9, A. pegreffii; 4, new genotype; 6 and 8, hybrid genotypes. Taq I: 2–6, and 12, A. pegreffii; 8, 10 and 13, A. simplex s.s.; 7 and 9, hybrid genotypes; 11, new genotype. 1 is always DNA molecular weight marker XIV (Roche).

(84) for the hybrids. Nm represents the gene flow between the populations and had a value of Nm = 15.22 between A. pegreffii and A. simplex s.s. In spite of the high number of genetic markers generated with RAPD-PCR, we did not observe any fixed allelic difference between the two species.

The gene flows between A. pegreffii and A. simplex s.s. and the new genotype were 0.63 and 0.66, respectively, and there could be a fixed allelic difference (M13-696 marker present in the new genotype and absent from A. pegreffii, A. simplex s.s. and from the recombinant genotypes).

Table 2
Nei’s genetic identity (above diagonal) and genetic distance (below diagonal) between A. pegreffii, A. simplex s.s., hybrid genotypes and the new genotype

<table>
<thead>
<tr>
<th></th>
<th>A. pegreffii</th>
<th>A. simplex s.s.</th>
<th>Hybrid genotypes</th>
<th>New genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. pegreffii</td>
<td>0.000</td>
<td>0.973</td>
<td>0.997</td>
<td>0.850</td>
</tr>
<tr>
<td>A. simplex s.s.</td>
<td>0.028</td>
<td>0.980</td>
<td>0.846</td>
<td>0.864</td>
</tr>
<tr>
<td>Hybrid genotype</td>
<td>0.003</td>
<td>0.020</td>
<td>0.997</td>
<td>0.850</td>
</tr>
<tr>
<td>New genotype</td>
<td>0.162</td>
<td>0.167</td>
<td>0.147</td>
<td>0.846</td>
</tr>
</tbody>
</table>
3.2.2. Analysis of the genetic variation between individual specimens of *A. simplex* s.l.

We estimated Nei’s genetic distances for the 42 individual specimens of *A. simplex* s.l. studied by the RAPD technique and, using these distances, we constructed a UPGMA dendrogram (Fig. 4) which showed that the anisakids form four genetic groups: A, B, C and D.

Group A was comprised by 17 L3 (10 Med-Mo, 4 Med-Le and 3 Atl-Hu) identified by PCR-RFLP as 13 *A. pegreffii* (13) and 4 recombinant genotypes. Group B was comprised by 10 L3 (4 Med-Le, 5 Med-Ma and 1 Atl-Cant), of which six were *A. pegreffii*, two *A. simplex* s.s. and two hybrid genotypes. Group C was comprised by 14 L3 (1 Med-Le, 2 Med-Ma, 2 Atl-Hu and 9 Atl-Cant), of which 13 corresponded to *A. simplex* s.s. and 1 to a hybrid genotype. Group D corresponded to an individual Med-Mo parasite that had the new genotype.

When we considered each of these populations as a genetic group and analyzed the genetic diversity within and between them we found the following: the percentage of polymorphic loci in groups A, B and C was 67.13% (96), 47.55% (68) and 68.53% (98), respectively; gene flow between group D and any other group was very low and ranged from \( N_m = 0.46 \) to 0.56. While 4.33, 3.79 and 3.05 were the \( N_m \) values between B–C, A–C and A–B, respectively (Table 3). Table 4 shows Nei’s genetic distance values between these populations and Fig. 5 shows the tree constructed from these and the bootstrap percentages.

### 4. Discussion

Population genetic structure in a species can be defined as the distribution of genetic variation among individuals sampled over different spatial scales [14]. The amount of genetic variability we detect depends “a priori” on the study method chosen to investigate the genetic structure of parasitic nematode populations. Isoenzyme electrophoresis is the most commonly used technique in nematodes, especially in anisakids. This technique, like others that directly study the DNA, such as PCR-RFLP or PCR-sequencing, shows the polymorphism of a discrete number of individual loci. In contrast, the RAPD technique is characterized by showing the variability of a large number of anonymous loci, theoretically distributed through the whole genome. In any case, most of the polymorphisms observed will be those with little or no functional consequences, since the genetic variation that confers fitness is rapidly fixed or eliminated [15].

In contrast to PCR-based techniques, one of the main limitations of isoenzyme electrophoresis techniques in genetic variability studies is the large amount of biological material required, which considerably limits the number of enzymatic loci that can be studied of the same larva, given its size. Although we do not show this in the results, we also studied the electrophoretic mobility of leucine aminopeptidase (two loci, LAP-1 and LAP-2) and phosphoglucomutase (two loci, PGM-1 and PGM-2) enzymes, and found a large allelic homogeneity between those larvae that displayed enzymatic ac-
Fig. 4. Dendrogram based Nei’s genetic distance (method UPGMA-modified from Neighbor procedure of Phylop version 3.5. adopted by POPGENE version 1.31) for the 42 individual specimens of A. simplex s.l. Ape is A. pegreffii, As is A. simplex s.s., H are hybrid genotypes, H* is the specimen showed a TaqI pattern characteristic of A. pegreffii and hybrid when the HinfI enzyme was used. Ng is the new genotype. Med is Mediterranean see: Med-Mo is Motril bay, Med-Ma is Malaga coast, Med-Le is Levante coast. Atl is Atlantic ocean: Atl-Cant is Cantabrian coast, Atl-Hu is Huelva coast.
Atlantic waters this declines to 20.00% (3 of 15) while A. sim-
neranean coasts are identified by PCR-RFLP as from blue whiting specimens captured on Spanish Mediter-
Mattiucci et al. (1997) [2] . However, a series of findings seem on the geographical distribution of both species reported by
plex s.s. represents 66.67% (10 of 15). This agrees with data
method, which preferentially amplifies variable regions of the
tivity. In contrast, with the RAPD-PCR technique, 96.50% of
A. simplex
by these authors for
 pegreffii
is based, is the reproductive isolation between both species,
tests established by the absence of hybrids and recombinant geno-
types in sympatric areas [2] . However, in this study the hybrid
types in sympatric areas [2] . However, in this study the hybrid
gene flow was very low, always below the threshold Nm = 1
reach (Dnei = 0.028), when previous studies in
the most diverse living being groups, vertebrates and inver-
tebrates, indicate that the genetic distances between species
that belong to the same genus often vary between 0.22 and
1.60 [11]; (iii) One of the main foundations on which the de-
scription of A. simplex s.s. and A. pegreffii as sibling species
is based, is the reproductive isolation between both species,
established by the absence of hybrids and recombinant geno-
types in sympatric areas [2] . However, in this study the hybrid
genotypes represent 16.67% of the parasites studied (7 of 42)
and are present in all the populations studied except for the
one from the Cantabrian coast.

The classical morphological concept of species still exists
today and is useful and necessary for parasite classification.
However, this concept has some limitations when there are
found to be sibling species that are frequent among the ne-
nematodes, which must be classified using the biological con-
cept of species. Sibling species must be distinguished using
biochemical and molecular techniques, such that the greater
the number of loci studied the closer we will get to the true
description. Since sympatric species occupy the same geo-
ographical area at the same time they have the opportunity to
hybridize. When this does not occur they can be described as “good species” because they are reproductively isolated.
Allopatric species occupy areas that are separate in space or
time. Since they do not have the chance to find similar species
to themselves it is not known whether or not these would be
able to hybridize with other allopatric species. Often, two
allopatric species are not reproductively isolated and in the
absence of barriers behave as a single species. This could
have been what occurred in the case of the populations of A.
simplex s.s. and A. pegreffii previously studied, which could
have occupied allopatric regions.

If we analyze the genetic variation between the 42 indi-
vidual specimens of A. simplex s.l. studied (Fig. 4) we find
that these can be separated into four genetic groups called A,
B, C and D, which include a mixture of specimens from the
different geographical populations, although with a predom-
nance of Mediterranean ones in A and B and Atlantic ones
in C. This situation should not surprise us if we consider the
high agility of intermediate/paratenic and definitive hosts of
A. simplex s.s. The values of genetic distance (Table 4) and
gene flow between A, B and C are compatible with the ex-
istence of three different populations (genetic populations),
with a smaller gene flow between the genetic populations A
and B.

On the other hand, the L3 specimen collected from blue
whiting captured in Motril Bay and identified both by PCR-
RFLP and by RAPD-PCR as a new genotype could represent
a new sibling species of the A. simplex complex although this
must be confirmed by finding other members of this “new
species”. This possibility was supported by the following
findings: (i) values of genetic distances between this new
genotype and populations A, B and C ranged between 0.164
and 0.176, and between 0.147 and 0.167 when we compared
them with A. simplex s.s., A. pegreffii and its hybrids; (ii)
gene flow was very low, always below the threshold Nm = 1
and ranging from 0.63-0.66 and 0.46-0.56, respectively; (iii)
Gst values were considerably higher when population D was
included (Table 3). When sibling species are in an early de-
velopmental stage all the loci can be polyphyletic or para-
phyletic, and none can be used as genetic markers to differen-
tiate populations. These should be exclusively distinguished
on the basis of gene frequencies and genetic distances. In the
case of parasite species long after separation, many loci show
fixed allelic differences and, in this case, when the species
are sympatric, they can be differentiated by only one marker
[18].
References


