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Population genetic analysis of *Anisakis simplex s.l.* and *Anisakis pegreffii* (Nematoda, Anisakidae) from parapatric areas and their contact zone

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ABSTRACT

Genetic markers (ribosomal DNA and mitochondrial DNA) were used for molecular dissection of the *Anisakis simplex sensu lato (s.l). complex* populations. Host fish were caught off Moroccan coasts, where only *Anisakis pegreffii* is present, the sympatric area comprising Spanish coasts, and the Little Sole Bank fishing area from Nordeast Atlantic Ocean where the only present species is *A. simplex sensu stricto(s.s.*). Sequence variations in the amplification products were then assessed indirectly by digestion with restriction endonucleases or directly by sequencing for 623 L3 larvae. The sequences were used to infer the relationships between the two species under study using various methodological approaches. We reveal the high genetic diversity of *Anisakis simplex s.s.* and *A. pegreffii* in both mitochondrial and nuclear genes. We detected 10 and 2 fixed differences between *A. simplex s.s* and *A. pegreffii* in the Cox2 and ITS1, respectively. We found a proportion of putative hybrids below 20% with similar figures on the Atlantic and Mediterranean coasts. Moroccan hybrids were more similar to *A. pegreffii* reflecting backcrosses between these mixed genotypes and his ancestor *A. pegreffii.* We discuss the possible interpretation of these putative hybrids.

1. Introduction

In the past, most of the controversy regarding the systematics of anisakids was due to the exclusive use of morphological characteristics of L3 larvae to distinguish between species, especially in cases where the existence of sibling species was suspected. Applying molecular techniques has helped to resolve this problem. One of these complex sibling species is *Anisakis simplex sensu lato* (*s.l.*), which is made up of 3 species, *Anisakis simplex sensu stricto* (*s.s.*), *Anisakis pegreffii* and *Anisakis berlandi,* distinguishable via isoenzymes and molecular markers (D'Amelio et al., 2000; Kijewska et al., 2000; Nadler and Hudspeth, 2000; Valentini et al., 2006; Mattiucci and Nascetti, 2008; Cavallero et al., 2011; Mattiucci et al., 2013). However, this differentiation is not so clear in areas where *A. simplex s.s.* and *A. pegreffii* are sympatric, as it occurs along the coasts of the Iberian Peninsula and Japan, where it is possible to find specimens with combined allozyme or genetic patterns between these two species (Abollo et al., 2003; Martín-Sánchez et al., 2005; Hermida et al., 2012; Cipriani et al., 2015). The detection of these putative hybrids has been the cause of some controversy in terms of its interpretation mainly due to the large recovery of larval forms and to the rare observation of adult hybrids. Recently, adult *A. pegreffii, A. simplex s.s.* and hybrid individuals of the two species have been

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recovered in sympatry in the same host specimen (*Stenella coeruleoalba*) found stranded on the Sicilian coast by Cavallero et al. (2011). Until now adult hybrids were described solely in *Balenoptera acutorostrata* from the Japanese waters (Umehara et al., 2006). Differential biomedical importance traits as pathogenic and allergenic potential have been demonstrated between both sibling species (Romero et al., 2013; Arcos et al., 2014) giving more value to the taxonomic differentiation of these species. This highlights the need for us to focus on apparent hybridization phenomena.

The genetic structure of populations of a species is revealed to us by the distribution of the genetic variation between specimens of that species over different spatial scales and is conditioned by the study method chosen. Natural hybridization between species has been recorded in many groups of organisms. These phenomena may have profound impact on the dynamics of populations and species. Our objective, achieved through the study of parasites taken from different hosts and various geographical origins where these species are sympatric and parapatric, was to contribute to the existing knowledge on the distribution of the genetic variation of these two *Anisakis* species, *A. simplex s.s* and *A. pegreffii*, and their putative hybrids.

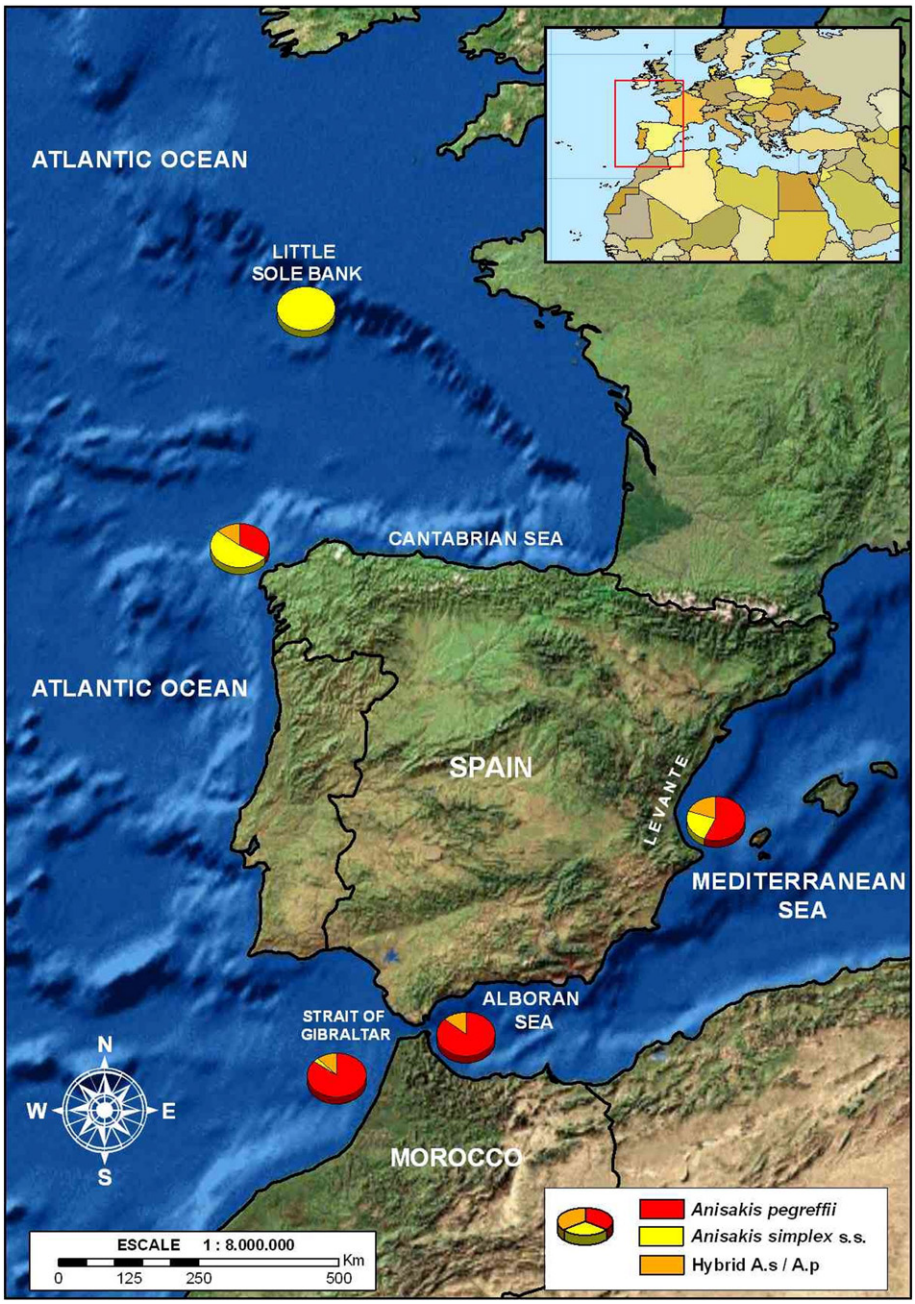

Fig. 1. Map of the area showing the geographical distribution of the different capture locations and the relative proportions of *A. simplex s.s.*, *A. pegreffii* and hybrids.

2. Materials and methods

2.1. Parasites, fish and geographical areas

Anisakis larvae were collected from mackerel (*Scomber japonicus*), horse mackerel *(Trachurus trachurus*), blue whiting (*Micromesistius poutassou*) and hake (*Merluccius merluccius*) caught at different points along the Moroccan coastline, off the coasts of the Iberian Peninsula and in the Northeast Atlantic (Little Sole Bank fishing ground) (Fig. 1). 30% of the larvae isolated from each fish were clarified in acetic alcohol (70% alcohol, 5% glacial acetic acid) and identified morphologically as type I L3 under a stereoscopic microscope. They were subsequently placed individually in appropriately labelled Eppendorf tubes and kept at −20 °C until molecular identification by Polymerase Chain Reaction–Restriction Fragment Length Polymorphisms (PCR-RFLP).

2.2. DNA extraction

Each larva 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 and genomic DNA was extracted using the RealPure kit for genomic DNA extraction by REAL (Ref RBMEG01). The precipitated pellet was resuspended in 20 μl of bidistilled water and kept at −20 °C until use.

Several precautions were taken to avoid cross-contamination between samples, including the use of separate rooms for DNA extraction processes as well as dividing reagents into small aliquots, frequent glove changes and the use of negative controls of the DNA extraction processes (a tube containing water every 6 sample tubes).

2.3. Molecular identification by PCR-RFLP

PCR amplification of the ITS1–5,8S-ITS2 of the rDNA was carried out using the primers A (forward), GTC GAA TTC GTA GGT GAA CCT GCG GAA GGA TCA and B (reverse), GCC GGA TCC GAA TCC TGG TTA GTT TCT TTT CCT, as described by D'Amelio et al. (2000) or NC5 (forward), 5′ GTA GGT GAA CCT GCG GAA GGA TCA TT 3′ and NC2 (reverse), 5′ TTA GTT TCT TTT CCT CCG CT 3′, as described by Zhu et al. (1998) adhering to the amplification conditions set out by the same authors. Briefly, 10 min at 94 °C, then 35 cycles of 30 s at 94 °C, 30 s at 55 °C (primers A/B) or 30 s at 60 °C (primers NC5/NC2) and 60 s at 72 °C, followed by a final elongation of 5 min at 72 °C.

RFLP was performed independently with three restriction enzymes, *TaqI* (5′...T↓CGA...3′) Fast Digest Thermo Scientific at 65 °C for 10 min, *HinfI* (5′...G↓ANT...3′) Fast Digest Thermo Scientific at 37 °C for 10 min, and *Cfo* (5′...GCG↓C...3′) Roche at 37 °C for 60 min, using a final enzyme concentration of 1 U. The results were visualised through electrophoresis in 3% agarose gel, which permitted the sibling species of *A. simplex* complex to be identified according to the band pattern. In *A. simplex s.s.*, digestion with *HinfI* enzyme produced two fragments of 620 and 250 bp as well as a weaker one of 100 bp; *TaqI* endonuclease provided three fragments: one of 430 bp, one of 400 bp and a weak one of 100 bp. The *A. pegreffii* larvae presented a pattern of three bands of 370,300 and 250 bp for *HinfI* enzyme and three of 400,320 and 150 bp for *TaqI* enzyme. For hybrid individuals, PCR-RFLP band pattern with the two restriction enzymes, *HinfI* and *TaqI*, is the sum of the patterns generated for *A. simplex s.s.* and *A. pegreffii*. *Cfo* endonuclease provided a pattern of two bands of 450 and 550 bp for the two sibling species.

2.4. PCR-sequencing and comparative sequence analysis

PCR amplification of the ribosomal fragment ITS1–5,8S-ITS2 was carried out with the A/B or NC5/NC2 primers, and of the mitochondrial Cox2 with the 210/211 primers and amplification conditions described by (Nadler et al. (2000)). The PCR products were purified using the Real Clean Spin Kit (Real) and then were directly sequenced in both directions using the primers used for DNA amplification. Sequences were edited and aligned to identify haplotypes (= unique sequences) using Clustal-X 1.81 software and manually adjusted, if necessary. When sequence ambiguities were found, we repeated the amplification and sequencing process in both directions. For a limited number of larvae with these ambiguities, we also carried out the random PCR clone sequencing.

Phylogenetic and phenetic analyses were performed using PHYLIP version 3.65 (http://evolution.genetics.washington.edu/phylip). The ITS1–5,8S-ITS2 and Cox2 sequences of *Anisakis typica* were used as outgroup. Various published sequences for both fragments in *A. simplex s.s.* and *A. pegreffii* have also been included in the study. We used the bootstrap as a measure of support or stability of the branches: a bootstrap percentage greater than or equal to 50% was considered sufficiently robust. The analysis was carried out using two different methods, parsimony analysis and analysis based on distance matrices; for the latter we used the F84 model of nucleotide substitution (the default method) with both NJ and UPGMA methods of clustering. The F84 model incorporates different rates of transition and transversion, and different frequencies of the four nucleotides; in addition to this, where one or both sequences contain at least one of the other ambiguity codons such as Y and R, the programme performs a maximum likelihood calculation.

For intra-specific analyses, statistical parsimony in TCS (v. 1.21) software was used. TCS is a Java computer programme to estimate gene genealogies including multifurcations and/or reticulations (i.e. networks) (Clement et al., 2000). This programme classifies ambiguous positions as lost data.

3. Results

3.1. Anisakis simplex s.l. collected and identified by PCR-RFLP

A total of 622 larvae have been identified by PCR-RFLP as *A. simplex s.s*., *A. pegreffii* or (As/Ap) mixed genotype. On the Spanish Mediterranean coasts, *A. pegreffii* represents 57.5% (23/40) of the L3 isolated from blue whiting, whilst 22.5% (9/40)were identified as *A. simplex s.s* and 20.0% (8/40) belonged to the category of As/Ap putative hybrids. On the Atlantic coast of Spain, the proportions were 32.8% (41/125), 52.0% (65/125) and 15.2% (19/125), respectively. In the Little Sole Bank fishing ground in the Northeast Atlantic, *A. simplex s.s*. (255/255) was the only species identified in hake. On both the Atlantic and Mediterranean coasts of Morocco, *A. pegreffii* was the dominant species in horse mackerel and mackerel (85.1%, 172/202); *A. simplex s.s*. only represented 1.9% of the anisakids collected from mackerel in the Atlantic (0.5% of the total, 1/202), and the rest (14.4%, 29/202) were As/Ap specimens (Fig. 1). 55.4% (31/56) of the putative hybrids only displayed this mixed genotype with one restriction enzyme, whilst with the other we observed a band pattern which was indistinguishable from *A. pegreffii.*

3.1.1. Comparative analysis of ITS1–5,8S-ITS2 and Cox 2 sequences

Comparative sequence analysis for the identification of haplotypes or unique sequences was only carried out on those larvae for which we had obtained the complete sequence of the fragment (860 bp), namely on 24 specimens of L3 larvae (Table 1). The number of haplotypes identified was 10 in *A. pegreffii*, and 4 in *A. simplex s.s* which were defined by 14 and 6 polymorphic sites respectively (Table 2). Only two fixed differences were detected between the two sibling species in positions 255 and 271 belonging to the ITS1 sequence (Table 2). We were also able to detect these differences in other specimens not included in Table 2 for which only a partial sequence of the fragment was obtained. In both positions it was a C/T transition; C in *A. pegreffii* and T in *A. simplex s.s*. The As/Ap specimen fully sequenced (Table 2) had C/T in 255 and T in 271 like other two not completely sequenced and for this reason not included in Table 2. The AT content of the sequences was 53.3% for *A. simplex s.s*. and 53.8% for *A. pegreffii.*

None of the 3 trees generated with ITS1–5,8S-ITS2 sequences, using both distance analysis methods (UPGMA and NJ) and MP, showed the separation between the two species, therefore the *A. simplex s.s* and *A. pegreffii* specimens appeared intermingled. The bootstrap values supporting the different branches were low, generally below 50% (data not shown).

In the 20 L3 specimens for which the complete Cox2 sequence was obtained, 11 *A. pegreffii,* 8 *A. simplex s.s* and 1 As/Ap according to PCR-RFLP results (Table 1), the comparative sequence analysis (547 bp) allowed us to identify 20 different haplotypes, one per specimen. Just as in the case of the rDNA, we did not include specimens for which only partial sequences of under the 547 bp indicated above were obtained. These haplotypes are defined by 59 polymorphic sites, 25 in *A. pegreffii* and 44 in *A. simplex s.s*. (Table 3). We detected the existence of 10 fixed differences between the two species, 9 transitions and one transversion (Table 3).

One of the L3 larvae from the Little Sole Bank fishing ground, identified by PCR-RFLP, and subsequently confirmed by the sequencing of this same fragment as *A. simplex s.s*. (rHS14), revealed a mitochondrial Cox2 gene sequence corresponding to *A. pegreffii* (mtHS19).

The AT content of the Cox2 gene sequence was 65.1% for both species, a lower percentage than that indicated by (Kim et al. (2006)) for the set of *A. simplex* mitochondrial genes (71.2%).

The topology of the Cox 2 trees constructed both by the two distance analysis methods (UPGMA and NJ) and by MP was very similar (Fig. 2). In all three cases, separation into two groups was observed (bootstrap values from 66.2 to 98.8%), one made up of

Table 1
Geographical origin and host of the 38 L3 larvae for which a complete sequence of the ITS1–5,8S-ITS2 (ITS) and/or Cox2 fragment was obtained. Every larva was previously identified by PCR-RFLP as *A. simplex s.s*., *A. pegreffii* or hybrid genotype; n is the number of L3 identified.

n	Species	Geographical origin	Host species	Markers
5	*A. pegreffii*	Atlantic coast of Morocco	*Scomber japonicus*	ITS
1	*A. pegreffii*	Atlantic coast of Morocco	*Scomber japonicus*	ITS/*cox2*
7	*A. pegreffii*	Atlantic coast of Morocco	*Trachurus trachurus*	ITS
2	*A. pegreffii*	Mediterranean coast of Morocco	*Trachurus trachurus*	ITS
1	*A. pegreffii*	Mediterranean coast of Morocco	*Scomber japonicus*	ITS
1	*A. pegreffii*	Mediterranean coast of Spain	*Micromesistius poutassou*	ITS/*cox2*
1	Hybrid genotype	Atlantic coast of Morocco	*Trachurus trachurus*	ITS
1	*A. simplex s.s*	Mediterranean coast of Spain	*Micromesistius poutassou*	ITS
1	*A. simplex s.s*	Atlantic coast of Spain	*Micromesistius poutassou*	ITS
1	*A. simplex s.s*	Atlantic coast of Spain	*Micromesistius poutassou*	ITS/*cox2*
3	*A. simplex s.s*	Northeast Atlantic (Little Sole Bank)	*Merluccius merluccius*	ITS/*cox2*
2	*A. pegreffii*	Atlantic coast of Morocco	*Scomber japonicus*	*cox2*
2	*A. pegreffii*	Atlantic coast of Morocco	*Trachurus trachurus*	*cox2*
3	*A. pegreffii*	Mediterranean coast of Morocco	*Trachurus trachurus*	*cox2*
2	*A. pegreffii*	Mediterranean coast of Morocco	*Scomber japonicus*	*Cox2*
1	*A. simplex s.s*	Mediterranean coast of Spain	*Micromesistius poutassou*	*Cox2*
2	*A. simplex s.s*	Atlantic coast of Spain	*Micromesistius poutassou*	*Cox2*
1	*A. simplex s.s*	Northeast Atlantic (Little Sole Bank)	*Micromesistius poutassou*	*Cox2*
1	Hybrid genotype	Mediterranean coast of Spain	*Micromesistius poutassou*	*Cox2*

Table 2
Alignment of the polymorphic positions detected in the ribosomal haplotypes identified. According to the PCR-RFLP identification, rHP are haplotypes of *A. pegreffii*, rHS are haplotypes of *A. simplex s.s.*, rHH is the hybrid genotype. (−) represents a deflection, Y = C/T, S = C/G.

Positions																							
	0	0	1	1	1	1	2	2	2	2	2	2	3	4	4	5	6	6	6	7	7	8	8
	0	3	4	6	7	7	5	5	7	9	9	9	0	2	6	7	0	0	4	0	4	0	2
Haplotypes	4	5	3	5	3	4	5	9	1	3	4	9	2	4	2	6	2	3	9	5	1	2	4
rHP1	G	G	G	C	G	C	C	T	C	C	T	G	C	C	G	C	G	T	A	C	T	A	A
rHP2																				S	C		
rHP3	C																T	C			C		
rHP4																						T	
rHP5															A								
rHP6												C									C		
rHP7														G							C		
rHP8			C	T	T	T				G	G										C		
rHP9																					C		
rHP10																					C		
rHS11							T		T														–
rHS12		C					T		T							A			T		C		
rHS13							T		T				A								C		
rHS14							T		T												C		
rHH15							Y	Y	T												C		

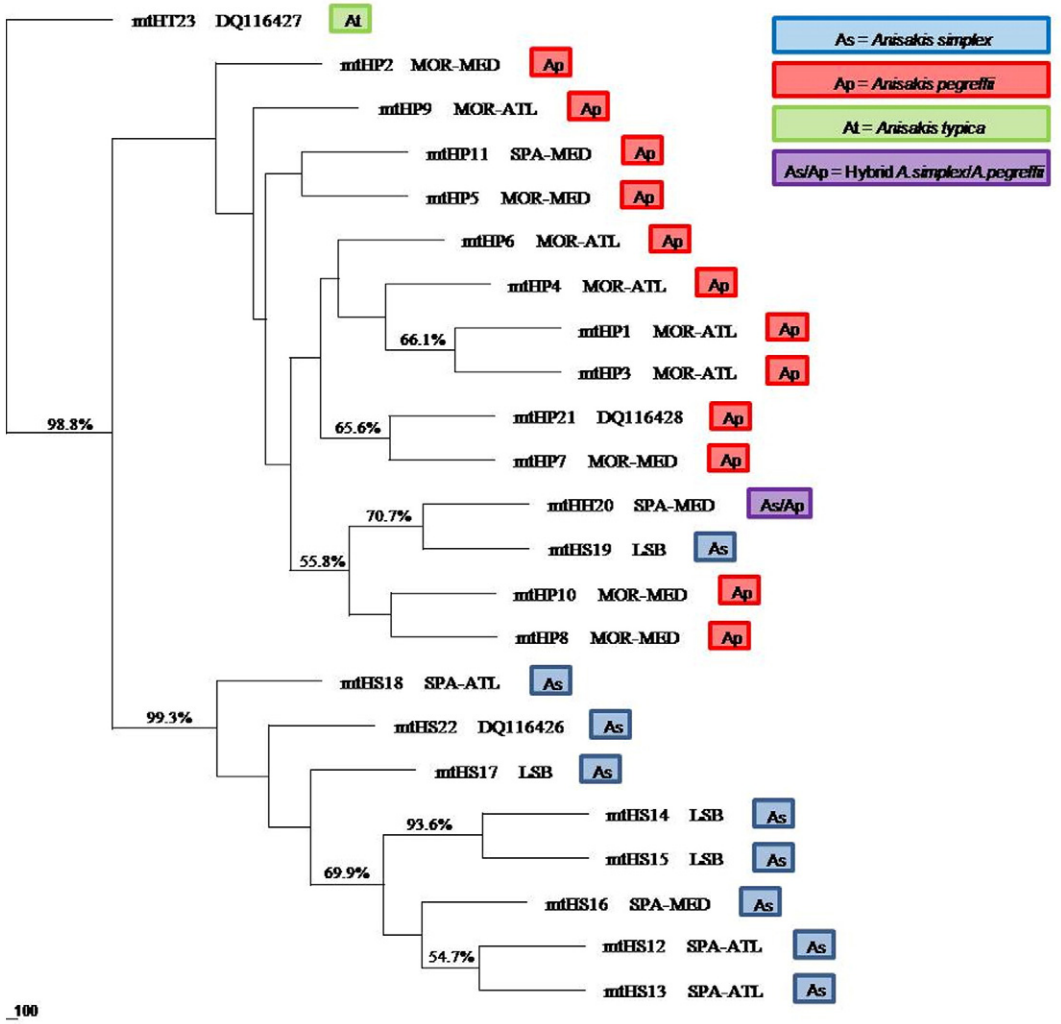

Fig. 2. Phenetic tree based on mtDNA Cox2 sequence data and obtained by distance analysis — F84 model with a neighbour-joining method of clustering. *Anisakis typica* is used as an outgroup. The numbers above the branches are bootstrap percentages (1000 replications) for clades supported above the 50% level.

all the haplotypes of *A. simplex s.s.* and the other by all those of *A. pegreffii*. The As/Ap specimen completely sequenced (mtHH20) was grouped together with *A. pegreffii* in the NJ and MP trees, whilst in the UPGMA tree it was independent from this group (bootstrap value of 99.8%), although it remained more closely related to *A. pegreffii* than to *A. simplex s.s.*

The ITS1–5,8S-ITS2 and Cox2 haplotype sequences were independently subjected to genealogy analyses by statistical parsimony. This algorithm sorted the *A. simplex s.l.* haplotypes into one ribosomal network (Fig. 3) and three independent mitochondrial networks (Fig. 4): 1 for *A. simplex s.s.*, another for *A. pegreffii* and a third one formed by the putative hybrid specimen. This programme collapses sequences into haplotypes and calculates the frequencies of the haplotypes in the sample; these frequencies are used to estimate haplotype outgroup probabilities, which correlate with haplotype age. In the ribosomal network, rHP9, from the Atlantic coast of Morocco, was the oldest ancestral haplotype (Fig. 3), from which the rest would have been derived by mutation. In the mitochondrial networks, mtHP3 in *A. pegreffii* and mtHS13 in *A. simplex s.s.*, both from Atlantic waters, were the oldest ancestral haplotypes (Fig. 4).

4. Discussion

Natural hybridization can occur between members of closely related species. There are many examples from the literature of gene crossing species borders on parasites and free-living organisms (Anderson, 2001). Hybrid zones are created in areas of contact between two parapatric species, where genetic exchange takes place to form hybrids.

A. simplex s.s. and *A. pegreffii* are sympatric on the coasts of the Iberian Peninsula. The southern limits of *A. simplex s.s.* in the Northeast Atlantic are the waters of the Straits of Gibraltar. Thus, we have found that this species only represents 0.5% of the larvae isolated from Moroccan coast. It was only found in *S. japonicus* from Atlantic waters revealing the migratory movements of this host from more northern latitudes. On the Spanish Mediterranean coast this species represents 22.5% (Fig. 1). We confirm that *A. pegreffii* is the dominant species in the western Mediterranean coasts of Spain and Morocco, where we found it in blue whiting, mackerel and horse mackerel. We also confirm that in Atlantic waters, the northern limits of its distribution area are the Iberian coasts (Mattiucci and Nascetti, 2008) hence we have not found it in Little Sole Bank fishery.

We found 16.4% rDNA ITS intermediate genotypes As/Ap in Spanish waters, in similar proportions ($p = 0.475$) on the Atlantic (15.2%) and Mediterranean (20.0%) coasts. Similar figures of mixed genotypes have been found in blackspot seabream from Portuguese waters (Hermida et al., 2012) and in sardines from Iberian waters (Molina-Fernández et al., 2015). This is not surprising considering that the two species often co-infected the same individual fish (both in the viscera and in the flesh) from Iberian waters (Umehara et al., 2006). Further south on the neighbouring coast of Morocco, putative hybrids constitute 14.9%. This is a similar figure to that found on Spanish coasts ($p = 0.592$) and there are no significant differences here between the Atlantic and the Mediterranean ($p = 0.575$) either. It is worth noting that even with such a low proportion of *A. simplex s.s.*, the percentage of intermediate genotypes As/Ap is similar. However, 75.9% (22/29) of the Moroccan hybrids only display this mixed genotype with one of the two restriction enzymes which permit differentiation between parent species (*Taq* or *Hinf*), whilst with the other they display a band pattern which is indistinguishable from *A. pegreffii*. Among Spanish individuals this occurs in lower proportions ($p = 0.001$); to be precise, in 33.3% (9/27). No putative hybrid was found which was more similar to *A. simplex s.s.* The

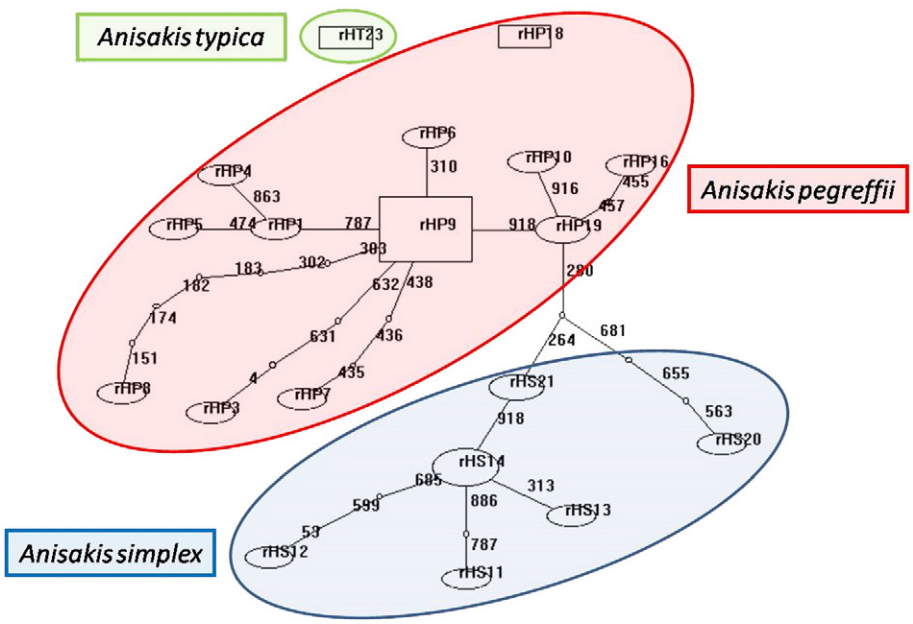

Fig. 3. Parsimony network based on rDNA ITS1–5,8S-ITS2 sequence data. The haplotype with the highest outgroup probability is displayed as a square, whilst other haplotypes are displayed as ovals. The size of the square or oval corresponds to the haplotype frequency.

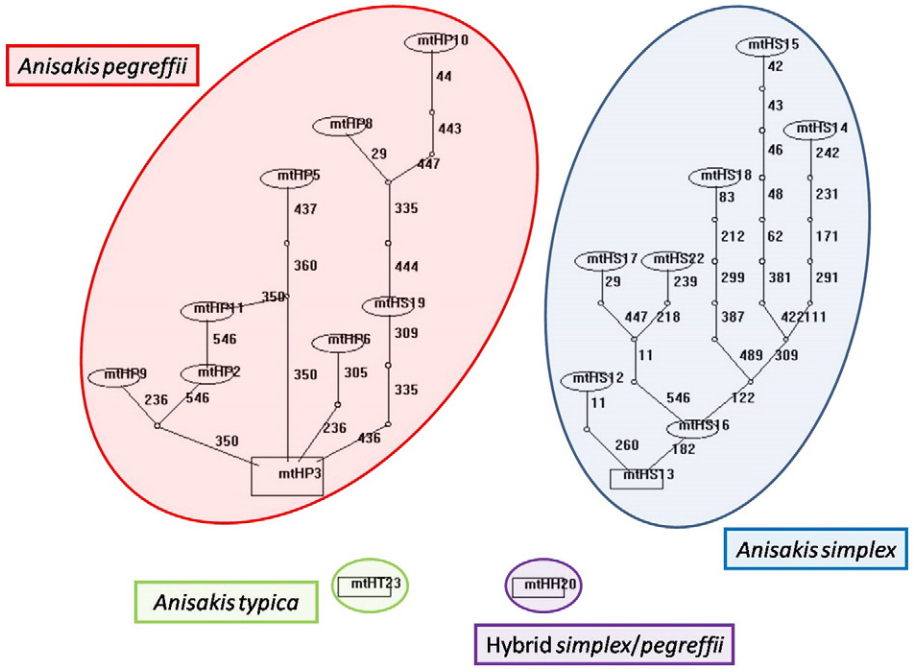

Fig. 4. Parsimony network based on mtDNA Cox2 sequence data. The haplotype with the highest outgroup probability is displayed as a square, whilst other haplotypes are displayed as ovals. The size of the square or oval corresponds to the haplotype frequency.

intermediate genotypes As/Ap are distinguished by having a DNA strand which is characteristic of *A. simplex s.s.* (therefore T, T in positions 255 and 271) and another strand characteristic of *A. pegreffii* (C, C) (Table 2). Backcrosses between these putative hybrid genotypes and *A. pegreffii* generate structures such as those seen in the complete (rHH15) or partially sequenced mixed specimens, which have C/T and T in these positions respectively. The PCR-RFLP band patterns obtained with some larvae suggest that in other cases there is a T in 255 and C/T in 271.

The identification of 14 ribosomal haplotypes reveals a considerable degree of intra-specific variability both in *A. simplex s.s.* and in *A. pegreffii*. However, as is to be expected due to its higher evolution rate, the genetic variability of the Cox2 gene is greater than that of the ribosomal genes. We detected 25 polymorphic sites in *A. pegreffii* and 44 in *A. simplex s.s.* (Table 3) which allowed us to identify a different haplotype for each specimen. Within *A. simplex s.s.,* mtHS13 and mtHS16, both Iberian, are the most similar haplotypes, whilst mtHS15 (Iberian) and mtHS18 (Little Sole Bank) are the most different, with 10 different base-pairs. However, it is within *A. pegreffii* where we observe the greatest differences between haplotypes, a factor which also occurs with the ITS1–5,8S-ITS2 fragment; thus, for example, rHP3 and rHP8 differ in 9 base-pairs. The topology of the 3 trees based on the Cox2 gene sequences shows us the separation of the two sibling species, whilst differences detected in the ITS1 are insufficient to determine this divergence and specimens of both species are intermingled. Similar results were obtained by Martín-Sánchez et al. (2005) with genomic markers generated by RAPD-PCR. However, it has already been pointed out that these traditional methods are based on certain assumptions which render them inadequate for intra-specific studies, which would instead be better represented through network approaches (Posada and Crandall, 2001) such as those shown here in the Figs. 3 and 4. As we can see, the ribosomal sequences were unsuccessful once again to separate the two species, forming a single network, although the *A. simplex s.s.* specimens are grouped together in one branch (Fig. 3). Based on our results, this is reflecting the introgression of the two species that is taking place in this area of sympatry. This natural hybridization between the two cryptic species originates individuals with mixed features that fish migration expands geographically. One of the larvae analysed from the Little Sole Bank fishery had mtDNA from *A. pegreffii* and rDNA from *A. simplex s.s.*, which therefore represents a case of mitochondrial introgression, the first to be described in the *Anisakis* genus. This molecular evidence suggests that hybridization between parasites from the two species is possible.

In natural populations, the ancestral haploytpes are expected to persist in the population and to be sampled together with their descendants. In the ribosomal network (Fig. 4), and *A. pegreffii* haplotype from the Atlantic coast of Morocco (rHP9) is indicated as the most ancestral one from which the rest of the *A. pegreffii* and *A. simplex s.s.* haplotypes have derived.

In the early stages following divergence of two taxa, the shared haplotypes are a result of retention of ancestral polymorphism. Only after sufficient time has elapsed sequences from each taxon do reach a stage of reciprocal monophyly (fixed differences). Only at this stage these sequences do become useful to distinguish members of the two populations, even though there might have been no gene flow for a long time (Anderson, 2001). It has been suggested that retention of ancestral polymorphisms might have led to the situation where As/Ap intermediate individuals are described with PCR-RFLP of the ITS1–5,8S-ITS2 fragment. Either of the two processes, hybridization or retention of ancestral polymorphisms, might have led to this situation.

Also, it is possible that both of these processes played a role. However, as evidenced by multilocus allozyme electrophoresis there are heterozygote genotypes at all of the diagnostic allozyme loci (Umehara et al., 2006) which seem to support the existence of hybridization in sympatric areas of the two cryptic species. Detection of differences in pathogenic and allergenic capacities of species of the *A. simplex* complex (Romero et al., 2013; Arcos et al., 2014) with higher values for *A. simplex s.s.*, support the taxonomic value of both species and highlight the importance of hybridization events.

5. Conclusions

In our opinion, *A. simplex s.s.* and *A. pegreffii* appear to be two parapatric species that continue to hybridise under natural sympatric conditions, reflecting the gradual and cumulative process of speciation. The Iberian coast would thus represent a bimodal hybrid zone between *A. simplex s.s.* and *A. pegreffii*, characterised by the coexistence of a low proportion of hybrids (<20%) together with two groups comprising the parental species. The results appear to indicate that backcrosses (crosses between F1 hybrids and one of the ancestors) may be relatively common especially with *A. pegreffii*, and in this way parental characteristics are recovered. We also reveal the high genetic diversity of *A. simplex s.s.* and *A. pegreffii* in both mitochondrial and nuclear genes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parepi.2016.02.003.

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