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Research paper

Investigating the genetic structure of the parasites *Anisakis pegreffii* and *A. berlandi* (Nematoda: Anisakidae) in a sympatric area of the southern Pacific Ocean waters using a multilocus genotyping approach: first evidence of their interspecific hybridization

Eleonora Bello [a,b,1], Marialetizia Palomba [b,c,1], Stephen C. Webb [d], Michela Paoletti [a], Paolo Cipriani [e], Giuseppe Nascetti [a], Simonetta Mattiucci [b,*]

[a] Department of Biological and Ecological Sciences, Tuscia University, Viale dell'Università snc, 01100 Viterbo, Italy
[b] Department of Public Health and Infectious Diseases, Section of Parasitology, Sapienza University of Rome, Laboratory affiliated to "Istituto Pasteur Italy - Fondazione Cenci-Bolognetti", P.le Aldo Moro 5, 00185 Rome, Italy
[c] Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Villa Comunale 1, 80121 Naples, Italy
[d] Cawthron Institute, Nelson, New Zealand
[e] Institute of Marine Research (IMR), Bergen, Norway

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A B S T R A C T

The southern Pacific Ocean, off the New Zealand coast, has been reported as one sympatric area of the two parasite species *Anisakis pegreffii* and *A. berlandi*. Here, a multilocus genotyping approach, based on a panel of eleven DNA microsatellite (SSR) loci *plus* the sequences analysis of the nuclear *nas10* nDNA and the mitochondrial mtDNA *cox*2 gene loci, was applied to a total of $N = 344$ adults and larvae of *Anisakis* spp. from cetacean and fish species, respectively. Out of the newly scored SSR loci, *Anisl* 15 and *Anisl* 2 showed fixed alternative alleles between *A. pegreffii* and *A. berlandi* resulting as 100% diagnostic loci. Out of SSRs *Anisl 00314* and *Anisl 7* previously disclosed, two additional loci, i.e., *Anisl* 4 and *Anisl* 22, were found to be sex-linked. The Bayesian genotypes clustering approach (STRUCTURE) allowed identification, with a 100% of probability value, $N = 208$ specimens to the "pure parental" *A. pegreffii*, $N = 133$ to the "pure parental" *A. berlandi*, while one adult and two larval stages showed mixed ancestry between the two groups having, in all cases, a Q-value = 0.50. NEWHYBRIDS analysis assigned (100% of probability) those specimens to their F1 hybrid category. This represents the first evidence of contemporary hybridization between the two parasite species in a sympatric area. The pairwise F_{ST} values estimated at intraspecific and interspecific level, inferred from both SSR loci and mitochondrial mtDNA *cox*2 sequences, have also demonstrated the existence of two distinct panmictic units in this study area, corresponding respectively to *A. pegreffii* and *A. berlandi*. The results obtained support the useful application of a multilocus approach in the identification of sibling species and their hybrid categories in sympatric areas. The possible use of sex-linked SSR loci of the two species of the *A. simplex* (s. l.), for sex determination of their larval stages, is also suggested.

1. Introduction

Nematode parasites of the genus *Anisakis* have a heteroxenous life cycle which involves various marine organisms at different levels of the trophic web in the marine ecosystem. The adults live in the stomach of marine mammals, mainly cetaceans (definitive hosts); fish and squid are intermediate or paratenic hosts, while planktonic or semi-planktonic crustaceans act as first intermediate hosts. To date, nine nominal species and two undescribed genotypes are recognised in the genus *Anisakis,* as based on genetic/molecular systematics (for a review, Mattiucci

* Corresponding author at: Department of Public Health and Infectious Diseases, Section of Parasitology, Sapienza - University of Rome, Italy Laboratory affiliated to "Istituto Pasteur Italy - Fondazione Cenci Bolognetti, P.le Aldo Moro 5, 00189 Rome, Italy.
E-mail address: simonetta.mattiucci@uniroma1.it (S. Mattiucci).
[1] Equal contribution.

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et al., 2018a). Indeed, the existence of nine species as distinct phylogenetic units has been demonstrated by various concatenated phylogenetic analyses, as inferred by combining both nuclear and mitochondrial sequences data (Valentini et al., 2006; Mattiucci and Nascetti, 2008; Cavallero et al., 2011; Mattiucci et al., 2014, 2018a). According to those analyses, four distinct clades within the genus *Anisakis* exist (Mattiucci et al., 2018a). Among them, one clade encompasses the three sibling species of the *A. simplex* (s. l.) complex: *A. simplex* (s. s.), *A. pegreffii* and *A. berlandi* (Nascetti et al., 1986; Mattiucci et al., 2014). Genetic/molecular markers mostly used for the recognition of the three sibling species of the *A. simplex* (s. l.) complex were inferred from mitochondrial gene loci (the direct sequences analysis of the mtDNA *cox*2) and nuclear loci, such as the allozymes (Mattiucci et al., 1997, 2016), the direct sequences analysis and the RFLPs-PCR of the ITS region of rDNA (D'Amelio et al., 2000), and the EF-1-alpha nDNA (Mattiucci et al., 2016); the last gene locus, however, was developed only in the species *A. pegreffii* and *A. simplex* (s. s.). More recently, the existence of those three species as distinct biological species, reproductively isolated, has been supported also using polymorphic DNA microsatellite loci (SSRs) (Mattiucci et al., 2019; Bello et al., 2020) and direct sequencing of the *nas10* nDNA (Palomba et al., 2020b).

Anisakis pegreffii has been so far recorded as a parasite, at the adult stage, of mainly oceanic dolphins belonging to the Families Delphinidae, Monodontidae and Phocoenidae and, less frequently, in Neobalenidae whales (Mattiucci et al., 2018a). At its third larval stage, it occurs in more than 60 pelagic and benthopelagic fish species, and in four squid species throughout its geographical range (Mattiucci et al., 2018a). *Anisakis pegreffii* is the most common species of *Anisakis* in the Mediterranean Sea, while in the Atlantic waters the northerly limit of its geographical range is represented by the Iberian coast, from where the species has been frequently identified in sympatry with *A. simplex* (s. s.) in several demersal and pelagic fish species as well as adults in cetaceans. However, *A. pegreffii* is also widespread in the Pacific Boreal region (Japan Sea, China Sea) and in the Austral Region, between 30°S and 60°S, where the species was identified at both adult and larval stages (reviewed in Mattiucci et al., 2018a; Gomes et al., 2020, 2021).

The species *A. berlandi* (see Mattiucci et al., 2014) currently exhibits a discontinuous distribution, being reported in the Southern Region, as in the Chilean Pacific Ocean, the South Shetland Islands, New Zealand waters and the South African Atlantic coast (Mattiucci and Nascetti, 2008; Klimpel et al., 2010; Mattiucci et al., 2018a). From these geographical areas, *A. berlandi* has been identified at the adult stage in six cetacean species, while its Type I larvae were so far identified in fish species from Southern waters from off New Zealand (Mattiucci et al., 2014, 2019; Bello et al., 2020), as well as from Southern Chilean (Mattiucci et al., 2018a) and Argentine (Irigoitia et al., 2018) coasts. Fourth-stage larvae of *A. berlandi* were also identified in *Kogia sima* from Australian waters (Shamsi et al., 2012) and, as L3, in other accidental hosts of the New Caledonian waters (Shamsi et al., 2017). Klimpel et al. (2010) stated that the occurrence of few larval specimens of *A. berlandi* and *A. pegreffii* in myctophids from the southern waters of the South Shetland Islands (Antarctic area) could be related to the introduction of those two parasites species from outside the Antarctic, through their migrating fish intermediate hosts. Accordingly, a very low occurrence of *A. berlandi* was recorded also in *M. leonina* from the South Shetland Islands (Mattiucci and Nascetti, 2008).

So far, the sympatric occurrence of *A. pegreffii* and *A. berlandi* is recorded in the South Pacific (i.e., in the New Zealand waters) (Mattiucci et al., 1997, 2014) and in the South Atlantic Ocean (Argentine coast) (Irigoitia et al., 2018), in Chile and South Africa (Mattiucci et al., 2018a). However, although their range of distribution overlaps in some geographic areas, the evidence of hybridization events between *A. pegreffii* and *A. berlandi* has not been so far recorded. It has been also indicated that nuclear multilocus genotyping approach should be used for investigating events such as hybridization, introgression and the retention of ancestral polymorphism in closely related anisakid species in sympatric areas (Mattiucci et al., 2016, 2018a). Thus, in recent years, in order to recognise parental genotypes of the species of the *A. simplex* (s. l.) complex and their mixed ancestry in known overlapping ranges of distribution, novel diagnostic markers from the nuclear genomes of these parasites have been developed (Mattiucci et al., 2016, 2019; Gómez-Mateos et al., 2020; Bello et al., 2020; Palomba et al., 2020b).

In the present paper, a multilocus genotyping approach, inferred from the screening of a panel of 11 microsatellite DNA loci (SSRs) and the sequences analysis of the *nas10* nDNA and the mtDNA *cox*2 gene loci, was carried out on adult and larval populations of *A. pegreffii* in comparison with populations of the sibling species *A. berlandi* detected in sympatry from one of their contact areas (i.e. New Zealand waters). The aim of this work is to: *i)* assign the specimens to the two parental taxa, i.e. *A. pegreffii* or *A. berlandi; ii)* recognise their mixed ancestry based on a Bayesian multinuclear clustering approach; and *iii)* infer data on the genetic diversity, at both nuclear and mitochondrial level, of the two species from this study area.

2. Materials and methods

2.1. Collection of parasite samples

A total of $N = 225$ adults (A) and fourth-stage larvae (L4) of nematodes from four cetacean species referred to the genus *Anisakis,* and $N = 119$ third-stage (L3) *Anisakis* Type I larvae (sensu Berland, 1961), collected from eight fish species from the South Pacific waters of the New Zealand coast, were genotyped by different genetic markers. Details concerning the life-history stage, definitive hosts (cetacean species) and intermediate/paratenic (fish species) of the *Anisakis* spp. specimens examined are given in Table 1. In particular, adult nematodes were from cetaceans stranded on the coast of New Zealand, i.e., three pilot whales, *Globicephala melas* (Traill), one Risso's dolphin, *Grampus griseus* (Cuvier), a Hector's dolphin, *Cephalorhynchus hectori* (van Beneden), and a common dolphin *Delphinus delphis* Linnaeus, 1758. The L3-stage larvae were collected from different fish species (Table 1) caught during the winter in 2018 along the southern coast of New Zealand.

The nematodes were repeatedly washed in saline solution and then preserved in 70% alcohol. Considering the sex-linkage of microsatellite loci, as discovered in our previous studies (Mattiucci et al., 2019; Bello et al., 2020), the adult specimens were first distinguished as females and males; in particular, sex discrimination was performed according to the main morphological diagnostic features between sexes (Mattiucci et al., 2014), using an optical microscope, at a total magnification of 100–400×. The numbers of adult female and male specimens studied are reported in Table 1. In the case of adult female worms, a tissue sample for molecular analysis was taken as distant as possible from the uterus to avoid possible contamination from stored male's sperm.

2.2. Genetic analysis of Anisakis spp. specimens by sequencing of nas10 nDNA and mtDNA cox2

All ($N = 344$) the specimens here studied were first assigned to their species level by the sequences analysis of the recently discovered as nuclear gene locus that is diagnostic between the three species of the *A. simplex* (s. l.) complex, i.e., the *nas*10 nDNA (Palomba et al., 2020b). Additionally, the same larval and adult specimens ($N = 344$) were also sequenced at the mtDNA *cox*2 gene locus, which is a standardised marker to recognise species of the genus *Anisakis* (Mattiucci et al., 2014).

Total DNA was extracted from a tissue portion (~2 mg) of each larva and adult specimen of *Anisakis* (Table 1). The extraction method by Quick-gDNA Miniprep Kit (ZYMO RESEARCH) was used. DNA obtained was quantified using a NanoDrop_TC1-E20 spectrophotometer (BioTek Synergy HT).

For the direct sequencing of the *nas*10 nDNA gene, the PCR amplification was performed according to the procedures detailed in Palomba

Table 1
Host species, life-history stage and sex of *Anisakis* specimens (*N*) collected in definitive and intermediate/paratenic hosts from off the New Zealand coast, genotyped at nuclear (*nas10* nDNA, SSRs and mitochondrial gene (mtDNA *cox*2) loci).

Hosts		*N*	N_A ♂♂	N_A ♀♀	N_{L4}	N_{L3}	*N nas10* nDNA	*N* SSRs	*N* mtDNA *cox2*
Definitive hosts									
Delphinidae	*Cephalorhynchus hectori*	3	1	2	–	–	3	3	3
	Delphinus delphis	28	9	13	6	–	28	28	28
	Globicephala melas	191	52	79	60	–	191	191	191
	Grampus griseus	3	–	–	3	–	3	3	3
Intermediate/Paratenic hosts									
Oreosomatidae	*Allocyttus sp.*	9	–	–	–	9	9	9	9
Centrolophidae	*Hyperoglyphe antarctica*	9	–	–	–	9	9	9	9
Trachichthyidae	*Hoplostethus atlanticus*	24	–	–	–	24	24	24	24
Merlucciidae	*Macruronus novaezelandiae*	25	–	–	–	25	25	25	25
Moridae	*Mora moro*	19	–	–	–	19	19	19	19
	Pseudophycis bachus	12	–	–	–	12	12	12	12
Gempylidae	*Rexea solandri*	10	–	–	–	10	10	10	10
Carangidae	*Trachurus declivis*	11	–	–	–	11	11	11	11
	Tot:	*344*	*62*	*94*	*69*	*119*	*344*	*344*	*344*

et al. (2020b), using the primers nas10-F (5′- GAT GTT CCT GCA AGT GAT TG −3′) and nas10-R (5′- CGC TAT TAA GAG AGG GAT CG - 3′).

For the sequencing of the mtDNA *cox*2 gene locus, PCR amplification was performed using the primers 211F (5′–TTT TCT AGT TAT ATA GAT TGR TTT YAT–3′) and 210R (5′–CAC CAA CTC TTA AAA TTA TC–3′). PCR conditions were established as previously described (Mattiucci et al., 2014).

2.3. PCR amplification of SSR markers and genotyping in Anisakis pegreffii and A. berlandi

The same specimens ($N = 344$) of *A. pegreffii* and *A. berlandi*, were also tested at the panel of seven SSR loci among those previously developed by Mattiucci et al., 2019 (i.e., *Anisl 00185, Anisl 00314, Anisl 10535, Anisl 05784, Anisl 08059* and *Anisl 00875)* and Mladineo et al., 2017 (i.e., *Anisl 7)* for the species *A. pegreffii* and *A. simplex* (s. s.) (Mattiucci et al., 2019), as well as in *A. berlandi* (Bello et al., 2020). Additionally, further four SSR loci (named as *Anisl 4, Anisl 22, Anisl 15* and *Anisl 2*), among those developed in Mladineo et al. (2017), were here tested for the first time on the collected specimens (Table 1).

The amplification of microsatellite loci was performed in three Multiplex PCRs: *Anisl 05784, Anisl 08059* and *Anisl 00875* in Multiplex 1; *Anisl 00185, Anisl 00314, Anisl 10535* and *Anisl 7* in Multiplex 2; finally *Anisl 4*, *Anisl 22*, *Anisl 15* and *Anisl 2* in Multiplex 3. Each Multiplex PCR amplification was performed in a 10 μl reaction volume, containing: 5–10 ng of genomic DNA, 5 μl Type-it Microsatellite PCR Kit (Qiagen®), double distilled water, and concentrations of 10 μM labeled forward and reverse primers each (Mattiucci et al., 2019). The following cycling protocol was used for the amplification for the three multiplex reactions: 35 cycles with 94 °C for 30 s, 56 °C for 90 s and 72 °C for 60 s. Before the first cycle, a prolonged denaturation step (95 °C for 15 min) was included and the last cycle was followed by a 15 min extension at 60 °C (Mattiucci et al., 2019).

Amplified PCR products were genotyped by an external Company (Macrogen service). The software Genemapper v.4.1 (Applied Biosystems, USA) was used to analyse the alleles obtained from the electropherograms. Genotyping errors generally associated with microsatellite analysis, such as stutter bands, the presence of null alleles and allelic drop-out were verified by using the software MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004).

2.4. Genetic data analysis

The sequences obtained at the *nas10* nDNA and the mtDNA *cox*2 gene regions from the $N = 344$ studied nematodes were aligned by using Clustal X (version 2.0) software (Larkin et al., 2007) with those of the same species previously obtained and deposited in GenBank (Mattiucci et al., 2014; Palomba et al., 2020b). This allowed the detection of those fixed diagnostic nucleotide positions that discriminate the species *A. pegreffii* and *A. berlandi*.

The observed heterozygosity (*Ho*), the expected heterozygosity (*He*), the mean number of alleles per locus (*A*) observed at the 11 SSR loci were calculated by ARLEQUIN version 3.5 (Excoffier and Lischer, 2010). The occurrence of the expected Hardy–Weinberg Equilibrium (HWE) at each SSR locus was assessed by means of exact tests, as implemented in the software ARLEQUIN version 3.5 (Excoffier and Lischer, 2010). Analysis of molecular variance (AMOVA), as implemented in ARLEQUIN version 3.5, with 1023 permutations, was applied on the genetic data sets obtained in the populations of *A. pegreffii* and *A. berlandi*. The fixation index (F_{IS}) from the genetic data sets obtained at the SSR loci in the analysed populations of the two *Anisakis* species, was estimated using ARLEQUIN version 3.5 (Excoffier and Lischer, 2010). The *Fst* (Weir and Cockerham, 1984) values, inferred from both SSR loci and mtDNA *cox*2 genetic data sets, were estimated by ARLEQUIN version 3.5 (Excoffier and Lischer, 2010).

To investigate instances of gene exchange between the two species, a Bayesian clustering algorithm was used from the SSRs and the *nas10* nDNA sequences data sets, performed by the program STRUCTURE v. 2.3.3 (Pritchard et al., 2000). The analysis was run setting the predefined number of clusters between 1 and 3. Twenty replicates of the analysis were carried out to check for consistency, each run for 100,000 MCMC iterations, following a burn-in of 50,000 iterations, under the admixture model and the assumption of correlated allele frequencies among populations. The best K value was identified using both the log probability of the data and the rate of change in the log probability of the data between successive K values as the optimality criteria (Evanno et al., 2005). The Bayesian analysis implemented in NEWHYBRIDS v. 1.1 (Anderson and Thompson, 2002) was also used to assess the occurrence of hybridization events between *A. pegreffii* and *A. berlandi*. This analysis assigns, probabilistically, each sampled individual to a predefined hybrid class, that is: pure parental *(A. pegreffii* or *A. berlandi*), first generation hybrid (F1), second generation hybrid (F2, or backcrosses). The assignment was performed on the SSRs and *nas10* nDNA data sets by computing 100,000 MCMC iterations, discarding the first 20,000 iterations as burn-in (after checking for stationarity), and replicating the analysis 10 times to check for consistency.

3. Results

3.1. Anisakis pegreffii and A. berlandi identification based on nas10 nDNA and mtDNA cox2 sequences analysis

A total of $N = 344$ larval and adult specimens of *Anisakis* spp. (Table 2) were identified by sequences analysis of the nuclear *nas10*

nDNA region. A fragment of 451 bp of the *nas10* nDNA region was obtained for all 344 specimens analysed. It revealed the presence of the nucleotide site diagnostic between the two species, as previously indicated in Palomba et al. (2020a). Indeed, at the position 251, a total of $N = 208$ individuals always showed the homozygote genotype G/G, while $N = 133$ specimens were always C/C homozygous (Fig. 1). According to this result, 208 specimens were assigned to the parental *A. pegreffii*, whereas 133 were assigned to the parental species *A. berlandi* (Table 2). Patterns of heterozygote genotypes (G/C), i.e., showing an overlapping peak at that diagnostic position (Fig. 1), were observed in three specimens (one adult collected from *G. melas* and two L3 larvae from *T. declivis*) (Table 2).

Sequences of the *nas10* nDNA region were deposited in GenBank under the accession numbers from MW691969 to MW691972 for *A. pegreffii*, and from MW691973 to MW691976, for *A. berlandi*.

The obtained sequences at the mtDNA *cox*2 locus (629 bp) in 208 specimens matched at the 99% or 100% the sequences *A. pegreffii* previously deposited by us in GenBank (Table 2), while $N = 136$ were assigned to *A. berlandi* species (Table 2).

Indeed, those 2 larval specimens from the fish host *T. declivis* and 1 female adult specimen from *G. melas*, which have shown a heterozygote genotype at the *nas10* nDNA, according to the mtDNA *cox*2 sequences resulted as belonging to *A. berlandi* (Table 2).

Sequences of the mtDNA *cox*2 region were deposited in GenBank under the accession numbers from MW658562 to MW658565 for *A. pegreffii*, and from MW658566 to MW658569, for *A. berlandi*.

3.2. Genetic diversity of SSR loci in A. pegreffii and A. berlandi

The same individual specimens ($N = 344$) previously sequenced at the nuclear gene *nas10* nDNA and mtDNA*cox2* gene regions, once genotyped at the eleven scored SSR loci, have shown some differences in the overall genetic diversity values in the two species, based on both the mean number of alleles per locus, and values of expected heterozygosity per locus (*He*) (Table 3).

Six loci (i.e., *Anisl 00185, Anisl 10535, Anisl 05784, Anisl 08059, Anisl 00875* and *Anisl 7*) were polymorphic in *A. pegreffii* and *A. berlandi*. Analogously, the newly tested SSR locus *Anisl 4* also was polymorphic in both parasite species. In contrast, the loci *Anisl 15* and *Anisl 2*, also here newly scored for the two *Anisakis* species, were found to be monomorphic. Additionally, in *A. berlandi*, only one allele was observed at the loci *Anisl 00314* and in the new tested *Anisl 22*; in contrast, the last locus showed 8 distinct alleles in *A. pegreffii* (Table 3).

The total number of alleles in *A. pegreffii* varied between 4, such as those observed at the locus *Anisl 7* in both larvae and adults, and the 14 found at the polymorphic locus *Anisl 08059* (Table 3). In the larval and adult populations of *A. pegreffii*, the mean value of alleles per locus (A), based on 11 loci, resulted as $A \approx 8.0$. In *A. berlandi*, the total number of alleles at the same loci was found to vary between 2, found in the loci *Anisl 10535* and *Anisl 05784*, up to 21 observed in the adult and larval specimens at the locus *Anisl 7* (Table 3). In *A. berlandi* the mean value of alleles per locus (A) was $A \approx 5,45$.

Deviation from the Hardy-Weinberg Equilibrium (HWE) at each locus was tested in the adult and larval populations of the two species (Table 3). No significant deviation between observed (*Ho*) and expected (*He*) heterozygosity was seen at the loci *Anisl 00185, Anisl 10535, Anisl 05784, Anisl 08059* and *Anisl 00875* in the analysed samples of *A. pegreffii* and *A. berlandi* (Table 3). In contrast, statistically high significant departures from the HWE in both larval and adult populations of *A. berlandi* were observed at the locus *Anisl 7,* and in the newly scored *Anisl 4.* Analogously, both adult and larval populations of *A. pegreffii* showed a significant deviation of *Ho* from the *He* at the newly scored loci *Anisl 4* and *Anisl 22*, as well as at the *Anisl 00314* and *Anisl 7* (Table 3).

3.3. New sex-linked SSR loci in A. pegreffii and A. berlandi

A positive F_{IS} value, indicating a marked excess of homozygotes from the expected HWE, was found at some SSR loci (Fig. 2a). In particular, significant positive F_{IS} values were recorded in *Anisl 00314, Anisl 7* and in the newly studied loci, *Anisl 4* and *Anisl 22* (Fig. 2). However, when considering only adult specimens and dividing the genotypes observed into adult male and female worms at the loci *Anisl 00314, Anisl 7, Anisl 4*

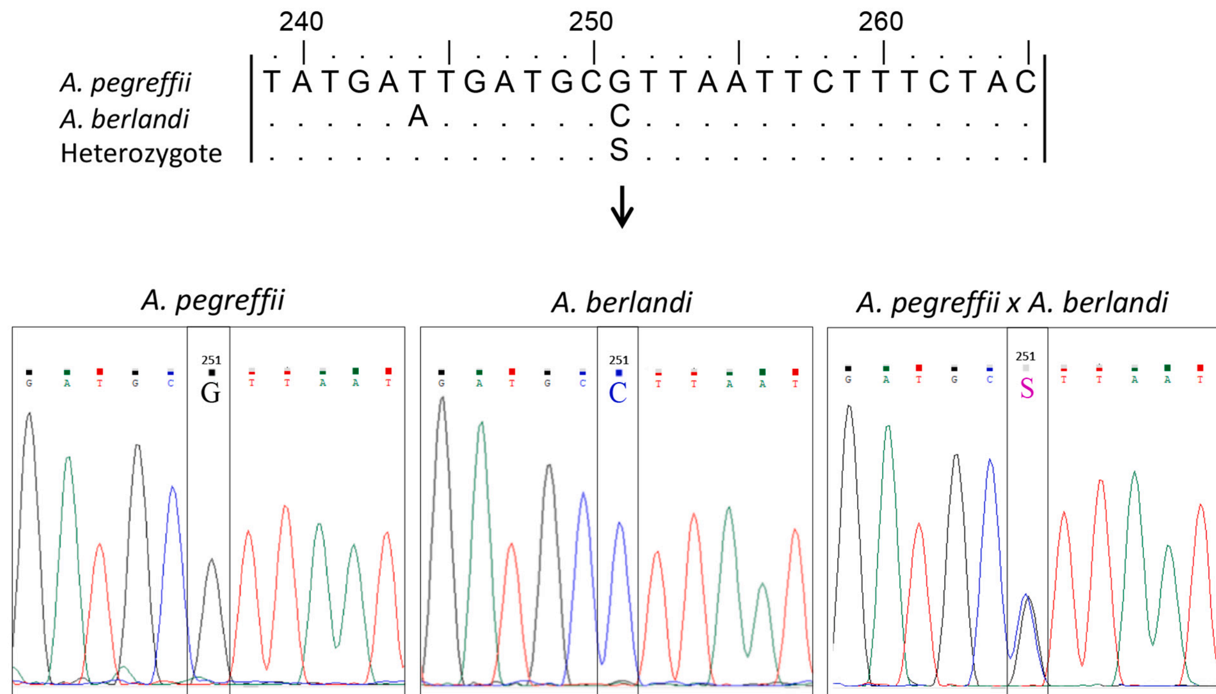

Fig. 1. Alignment of the *nas10* nDNA gene locus for the two species *A. pegreffii* and *A. berlandi*. The arrows show the fixed diagnostic nucleotide position detected between the two species. The heterozygote pattern at the diagnostic nucleotide position is shown. Dots indicate identity; standard IUPAC codes were used, i.e., S = C/G.

Table 2
Assignment of the *Anisakis* specimens tested in the present study to the parental taxa *Anisakis pegreffii* (Ap) or *A. berlandi* (Ab), or to mixed ancestry between them (Heterozygote), according to their genotypes observed at nuclear (i.e. *nas10* gene and SSRs loci) and mitochondrial (mtDNA *cox*2) markers.

Host species	*N*								
		nas10 nDNA			SSRs			mtDNA *cox*2	
Definitive Hosts	$N_{A,\ L4}$	Ap	Ab	Heterozygote	Ap	Ab	Heterozygote	Ap	Ab
Cephalorhynchus hectori	3	3	–	–	3	–	–	3	–
Delphinus delphis	28	28	–	–	28	–	–	28	–
Globicephala melas	191	137	53	1	137	53	1	137	54
Grampus griseus	3	3	–	–	3	–	–	3	–
Intermediate/Paratenic Hosts	N_{L3}								
Allocyttus sp.	9	–	9	–	–	9	–	–	9
Hyperoglyphe antarctica	9	–	9	–	–	9	–	–	9
Hoplostethus atlanticus	24	–	24	–	–	24	–	–	24
Macruronus novaezelandiae	25	3	22	–	3	22	–	3	22
Mora moro	19	8	11	–	8	11	–	8	11
Pseudophycis bachus	12	9	3	–	9	3	–	9	3
Rexea solandri	10	8	2	–	8	2	–	8	2
Trachurus declivis	11	9	–	2	9	–	2	9	2
Tot:	*344*	*208*	*133*	*3*	*208*	*133*	*3*	*208*	*136*

and *Anisl 22* in *A. pegreffii,* as well as *Anisl 7* and *Anisl 4* in *A. berlandi*, the F_{IS} value in the male worms of the two species was equal to 1, showing a homozygous genotype (Fig. 2b). Therefore, out of the loci *Anisl 00314* and *Anisl 7*, already known to be sex-linked loci in both species, also the loci *Anisl 4* and *Anisl 22* were seen to be sex-linked because of the hemizygosity state of the males in both the *Anisakis* spp., as X-linked inheritance. Indeed, the HWE again calculated at sex-linked loci considering only adult females of the two species, i.e., $N = 71$ of *A. pegreffii* and $N = 22$ of *A. berlandi*, showed no significant deviations from the HWE expectations (Table 4).

3.4. Multi-locus genotyping approach to distinguishing the two parental species and their hybrids

Allele frequencies estimated in *A. pegreffii* and *A. berlandi* are reported in Table 5. As regards the sex-linked loci (i.e., *Anisl 00314*, *Anisl 7, Anisl 4* and *Anisl 22*), the allele frequencies were calculated considering the hemizygosity of male specimens, according to Mattiucci et al. (2019): male specimens were considered as monoallelic, while their female counterparts were considered as biallelic. Among the 11 SSR loci considered, *Anisl 10535*, *Anisl 057*84, *Anisl 00875*, *Anisl 7* and *Anisl 22* have shown a differential frequency distribution at some alleles found in common in the two species (Table 5). Instead, at the loci *Anisl 15* and *Anisl 2*, $N = 208$ specimens of *A. pegreffii* were monomorphic, showing fixed alternative alleles (Table 5) with respect to those alleles observed in the specimens ($N = 133$) of *A. berlandi*. Indeed, the loci *Anisl 15* and *Anisl 2* were 100% diagnostic between the two parasite species (Fig. 3).

Heterozygote genotypes formed by those two fixed alternative alleles at the diagnostic loci *Anisl 15* and *Anisl 2* were observed in three specimens (Fig. 3): they were the same individual worms (Table 2) which have shown also a heterozygous genotype (C/G) at the diagnostic nucleotide site of *nas10* nDNA locus between the two parasite species (Fig. 1); those three individuals, at the other partially diagnostic SSR loci (i.e., *Anisl 10535, Anisl 05784, Anisl 00875, Anisl 7,* and *Anisl 22)* have shown a genotype including the most common alleles found at those loci in the two parental species. The peaks representing the alleles found at those 100% diagnostic SSR loci in the parental taxa and in the three heterozygote specimens are reported in Fig. 3. Additionally, because the three larval specimens have shown a heterozygote genotype, also at the polymorphic sex-linked loci *Anisl 4, Anisl 7* and *Anisl 00314,* they were assumed to be female worms.

According to the Bayesian clustering algorithm performed by STRUCTURE 2.3.3 (Fig. 4A), inferred from their genotypes recorded at those 100% diagnostic (i.e., *Anisl 15, Anisl 2*), and the partially diagnostic SSR loci (i.e., *Anisl 10,535, Anisl 05784* and *Anisl 00875*), *plus* those recorded at the *nas10* nDNA with the K = 2 clustering option, implemented by the Evanno method, which best fitted the data set ($\Delta K = 12.80$) (Supplementary Fig 1), a total of $N = 208$ specimens and $N = 133$ were assigned to the parental clusters (*A. pegreffii* and *A. berlandi* respectively) with high probability scores (Q-value = 100%). Of course, in this STRUCTURE analysis, the other partially diagnostic sex-linked loci (i.e., *Anisl 7* and *Anisl 22*) were excluded because of the hemizygosity of the male specimens. The three female nematodes have shown sign of mixed ancestry between the two groups having, in all cases, a Q-value = 50% (Fig. 4A). Finally, the analysis with NEWHYBRIDS identified with high level of confidence most of the individuals as 'pure' parental individuals of *A. pegreffii* or *A. berlandi* (Fig. 4B); the only exceptions were just those 3 individuals identified as admixed by the previous STRUCTURE analysis; according to NEWHYBRIDS they were assigned to the F1 hybrid class, with a 100% of probability value.

3.5. Intraspecific genetic differentiation of A. pegreffii and A. berlandi

Excluding the sex-linked loci because of the hemizygosity of males, the AMOVA analysis based on seven SSR markers showed that in both the species most of the variance was significantly allocated within individuals (≈96% and ≈97%). Further, a notable variation was found among individuals within populations, with $F_{IT} = 0.04$ ($p = 0.0009$) and $F_{IT} = 0.03$ ($p = 0.27$) in *A. pegreffii* and *A. berlandi,* respectively. (Table 6). Based on those SSR loci considered, a significantly high level of genetic differentiation has been found at the interspecific level, between the populations of *A. pegreffii* and those of *A. berlandi* (on average, $Fst \approx 0.605$, $p < 0.001$). In contrast, at the intraspecific level, lower and statistically not significant values were observed between metapopulations of larvae collected from fish species versus adult metapopulations of the two parasite species, recovered from cetacean hosts, being, $Fst \approx 0.002$ ($p = 0.21$) in *A. pegreffii,* and $Fst \approx 0.003$ ($p = 0.22$) in *A. berlandi*.

Analogously, considering the sequences analysis of the mitochondrial gene locus mtDNA *cox*2, the genetic differentiation among parental populations of *A. pegreffii* larvae and those adult conspecifics was, on average, $Fst \approx 0.01$ ($p = 0.10$). A similar value, i.e., $Fst \approx 0.02$ ($p = 0.12$), was observed at the mitochondrial level, between parental populations of *A. berlandi* larvae with respect to adult samples analysed in the present study. Conversely, higher statistically significant pairwise comparison value at the mtDNA *cox*2 sequences was recorded at the interspecific level between *A. pegreffii* and *A. berlandi* (i.e., on average, $Fst \approx 0.66$, $p < 0.001$).

Table 3
Genetic diversity at nine microsatellite loci of adult and larval specimens in populations of *A. pegreffii* and *A. berlandi* analysed in the present study. *Anisl 2* and *Anisl 15* were excluded from the estimates because they are monomorphic in both the species. Similarly, no estimates were possible for the loci *Anisl 00314* and *Anisl 22* loci because they are monomorphic in both larvae and adults of *A. berlandi*, as well as in *Anisl 10535* locus because it was monomorphic in adult and L4 specimens.

		A. pegreffii		*A. berlandi*	
		A + L4	L3	A + L4	L3
Locus					
Anisl 00185	*N*	171	37	53	80
	Ho	0.75	0.76	0.74	0.77
	He	0.81	0.79	0.83	0.84
	p	*0.61*	*0.73*	*0.51*	*0.33*
	A	9	7	11	9
Anisl 00314	*N*	171	37	53	80
	Ho	0.45	0.35	–	–
	He	*0.75*	*0.74*	–	–
	p	***	***	–	–
	A	8	6	1	1
Anisl 10535	*N*	171	37	53	80
	Ho	0.73	0.65	–	0.01
	He	0.76	0.79	–	0.01
	p	*0.45*	*0.55*	–	*1.00*
	A	9	8	1	2
Anisl 05784	*N*	171	37	53	80
	Ho	0.81	0.81	0.07	0.02
	He	0.79	0.77	0.07	0.02
	p	*0.09*	*0.31*	*1.00*	*1.00*
	A	12	9	2	2
Anisl 08059	*N*	171	37	53	80
	Ho	0.83	0.86	0.41	0.30
	He	0.86	0.87	0.36	0.26
	p	*0.05*	*0.66*	*0.84*	*0.45*
	A	14	12	4	3
Anisl 00875	*N*	171	37	53	80
	Ho	0.44	0.49	0.07	0.11
	He	0.49	0.42	0.07	0.11
	p	*0.28*	*1.00*	*1.00*	*1.00*
	A	8	6	3	4
Anisl 7	*N*	171	37	53	80
	Ho	0.21	0.13	0.56	0.47
	He	*0.25*	*0.33*	*0.91*	*0.92*
	p	***	***	***	***
	A	4	4	21	23
Anisl 4	*N*	171	37	53	80
	Ho	0.53	0.43	0.43	0.35
	He	*0.82*	*0.84*	*0.82*	*0.85*
	p	***	***	***	***
	A	12	12	14	15
Anisl 22	*N*	171	37	53	80
	Ho	0.41	0.32	–	–
	He	*0.61*	*0.62*	–	–
	p	***	***	–	–
	A	8	5	1	1

N, number of genotyped specimens at each locus; *Ho*, observed heterozygosity; *He*, expected heterozygosity; A, number of alleles detected at each locus; *p*, significance ($p < 0.05$) of the deviation from HWE expectation. ***$p \ll 0.001$.

4. Discussion

4.1. Nuclear markers to recognise parental *A. pegreffii* and *A. berlandi*, and their hybrid categories

In the present study, the testing of new SSR loci in species definition between *A. pegreffii* and *A. berlandi* has disclosed two further nuclear loci (i.e., *Anisl 15* and *Anisl 2*) that were 100% diagnostic between the two species. These loci can identify both male and female worms of the two parasite taxa, at any developmental stage. The finding of these two loci having a high diagnostic power acquires particular importance in the discrimination analysis of the two parasite species, especially in sympatric areas. Indeed, among the SSR loci we had previously tested in Mattiucci et al. (2019), only one, i.e., the locus *Anisl 7,* was indicated as able to discriminate *A. berlandi* from *A. pegreffii* (Bello et al., 2020). However, since *Anisl 7* is a sex-linked locus, it cannot be included in a multigenotyping approach inferred by STRUCTURE analysis, to identify clusters of the two parental taxa; furthermore, it is not useful in recognising the potential occurrence of "male hybrid genotypes" between the two parental taxa. Conversely, the newly assessed loci *Anisl 15* and *Anisl 2,* were found to be fixed for two alternative alleles in both two species. These two loci were thus successfully used in the assignment of 208 *A. pegreffii* and 133 *A. berlandi* specimens with a 100% score value to their specific parental clusters, as well the 3 heterozygote specimens to their F1 hybrid category. The usefulness of the loci *Anisl 15* and *Anisl 2,* here investigated for the first time, were validated over a large number of specimens. They were among those previously developed by Mladineo et al. (2017). These authors, however, did not recognise the discrimination power of those markers between the species of the *A. simplex* (s. l.) complex, nor their validity in disclosing hybrid categories and, even less, the presence of sex-linked loci. Indeed, in the last concerns, sex-linked loci SSR loci could be often erroneously considered as having null alleles, which is one among the reasons of significant departure from the HWE expectations when analysing the genetic variation inferred from DNA microsatellite loci (Criscione et al., 2007a; Mattiucci et al., 2019).

The present genetic analysis has once again underlined the importance to use a multi-genotyping approach in the discrimination of parental taxa and admixtured genotypes in a sympatric area of closely related species of *Anisakis*. Furthermore, this study has permitted a further validation of the nuclear gene locus *nas10* nDNA diagnostic power over a large number of larval specimens of the two parasite species. Indeed, in our previous study, the development of this nuclear marker was only investigated in adult specimens of *A. pegreffii* and *A. berlandi*, using the PCR-ARMS methodology based on the diagnostic positions detected as the *nas10* nDNA gene in the three species of the *A. simplex* (s. l.) complex (Palomba et al., 2020b). Here, the 344 specimens of *Anisakis* genotyped at the *nas10* nDNA, included in the Bayesian approach, produced complete agreement with the results obtained by the microsatellite loci. This congruency of these genetic markers permitted to define the population structure, in the contact zone, of the two parasite species, showing the existence of two distinct genetic clusters formed by the two parental species (i.e., *A. pegreffii* and *A. berlandi*), and providing clear evidence for their admixtured genotypes (F1 hybrids *A. pegreffii* x *A. berlandi*) (Fig. 4 A, B). Thus, the present study represents the first demonstration of the occurrence of hybridization events of *A. pegreffii* and *A. berlandi* in a contact zone. Indeed, while interbreed events have been frequently reported in sympatric populations of *A. simplex* (s. s.) and *A. pegreffii* (Abollo et al., 2003; Marques et al., 2006; Umehara et al., 2006; Lee et al., 2009; Suzuki et al., 2010; Chou et al., 2011; Molina-Fernández et al., 2015; Cipriani et al., 2015; Mattiucci et al., 2016; Gómez-Mateos et al., 2020), no hybridization or introgression phenomena had been so far reported between *A. simplex* (s. s.) and *A. berlandi* in contact and overlapping areas of their range of distribution (Mattiucci and Nascetti, 2007; Baldwin et al., 2011).

However, the finding of three F1 specimens of *A. pegreffii* and *A. berlandi* does not mean that the two taxa do not represent two "true biological species". In fact, the presence of only F1 hybrid genotypes does not imply that gene flow occurs between the two taxa, or that an incomplete speciation event had happened. It can instead indicate that reproductive isolation mechanisms may not be completely efficient for such related species. Pre-zygotic barriers for parasites include ecological isolation, isolation by host-specificity, as well as mate recognition systems (Southgate et al., 1998). In this regard, it seems that the first two conditions are not valid for *A. pegreffii* and *A. berlandi,* sharing both geographic and definitive hosts range (Mattiucci et al., 2018a). Thus, it might be supposed that chemical cues and/or other segregative mating recognition systems between conspecific adult specimens might not be

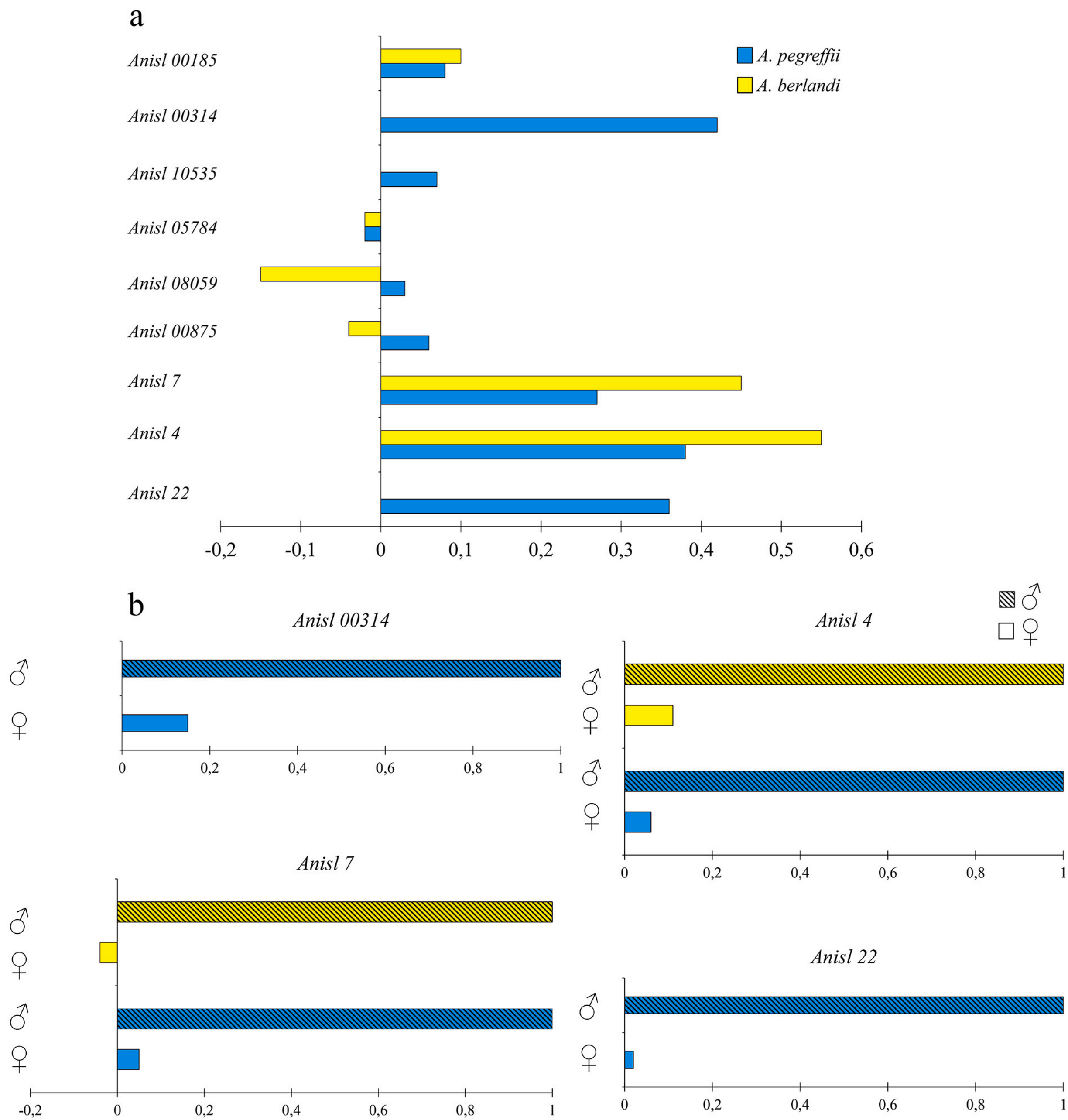

Fig. 2. (a) F_{IS} value calculated at nine microsatellite loci (except *Anisl 15* and *Anisl 2*, because of the monomorphism in both the species) studied in the two species *A. pegreffii* and *A. berlandi.* (b) F_{IS} in male and female adult specimens at the sex-linked loci in *A. pegreffii* (i.e., *Anisl 00314, Anisl 7, Anisl 4* and *Anisl 22*) and in *A. berlandi* (i.e., *Anisl 7* and *Anisl 4*). Negative values indicate heterozygous excess, while positive values indicate homozygous excess from that expected under Hardy–Weinberg Equilibrium (HWE).

highly efficient when syntopic infection in the same definitive host occurs. It can be also supposed that the absence of conspecific pairing partners, and mating stimuli for females of the rarer species in the syntopic situation may be important factors in increasing the likelihood of interspecific current hybridization (Avise and Saunders, 1984). Thus, a tendency for hybridization would take place preferentially between parental species of parasites differing greatly in abundance. This could be the case for anisakid nematodes infecting the same definitive hosts in a contact zone. Indeed, taking into consideration that the number of mature females of anisakid parasites is generally higher than that of mature males in their definitive hosts (Ugland et al., 2004), when the intensity of the infection by one of these two species is low, mating events between the two species could occur, resulting in a first generation (F1) of larval hybrids (Mattiucci et al., 2016). In support of this hypothesis, considering the cetacean hosts here analysed, the occurrence of adult worms of *A. berlandi* resulted lower with respect to those of the species *A. pegreffii* (Table 2). In addition, the F1 hybrids identified here have shown an *A. berlandi* maternal inheritance at the mtDNA *cox*2 gene sequences analysis.

The frequency of current hybridization events between *A. pegreffii* and *A. berlandi* so far observed in this contact zone of the Pacific Ocean appears to be around 1%. Higher frequency (around 1,6%) of contemporary hybridization has been reported in the sympatric area of the Atlantic Iberian coast between the species *A. pegreffii* and *A. simplex* (s. s.) (Cipriani et al., 2015; Mattiucci et al., 2016). However, according to available data, the percentage of F1 adult worms between species of the *A. simplex* (s. l.) complex is far lower with respect to the number of F1 larval stage observed; indeed, only few hybrid adult worms were

Table 4
Genetic diversity at the four sex-linked loci (i.e. *Anisl 00314*, *Anisl 7*, *Anisl 4* and *Anisl 22*) in *A. pegreffii* and *A. berlandi*, estimated in adult female specimens. Estimates at the *Anisl 00314* and *Anisl 22* loci were not possible because they are monomorphic in *A. berlandi*. *N* = number of genotyped specimens at each locus; *Ho*, observed heterozygosity; *He*, expected heterozygosity; *p*, significance ($p < 0.05$) of the deviation from HWE expectation. ***$p \ll 0.001$.

		A. pegreffii	*A. berlandi*
		♀♀	♀♀
Locus			
Anisl 00314	*N*	71	22
	Ho	0.67	–
	He	0.77	–
	p	*0.17*	–
Anisl 7	*N*	71	22
	Ho	0.30	1.00
	He	0.30	0.87
	p	*0.67*	*0.51*
Anisl 4	*N*	71	22
	Ho	0.79	0.77
	He	0.85	0.87
	p	*0.63*	*0.60*
Anisl 22	*N*	71	22
	Ho	0.61	–
	He	0.63	–
	p	*0.27*	–

detected (Umehara et al., 2006; Cavallero et al., 2014). In those studies, a total of 5 hybrid mature female worms were found. The two larval specimens with F1 hybrid patterns here detected were female nematodes (i.e., showing heterozygote genotypes at the sex-linked loci), co-infecting the same individual fish host with parental specimens of both *A. pegreffii* and *A. berlandi*. This would suggest that some selective factors would decrease the F1 hybrid fitness, impeding the full development to mature adults worms in the definitive hosts of those larval F1 specimens, at the same rate of the parental species. Hybridization may give rise to F1 hybrids with low fertility or progeny's viability, preventing gene flow between parental taxa. The results so far obtained seem to reject the scenario of viable, i.e., fertile and able to reproduce F1 hybrids between the two taxa, because no backcross genotypes were identified in this study. Thus, the hybridization between *A. pegreffii* and *A. berlandi* could be evaluated as a sporadic and contemporary phenomenon.

Other examples of contemporary hybridization have been detected between *Ascaris lumbricoides* and *A. suum,* just discovered by a Bayesian clustering approach based on microsatellite data in sympatric populations from Guatemala and China (Criscione et al., 2007b; Detwiler and Criscione, 2010), as well as in other cryptic parasite species, such as *Paramacrostrongylus* spp. (Chilton et al., 1997), *Fasciola hepatica* and *F. gigantica* (Agatsuma et al., 2000; Lin et al., 2007; Le et al., 2008; Peng et al., 2009; Calvani and Šlapeta, 2020), *Schistosoma mansoni* and *S. rodhaini* (Morgan et al., 2003; Steinauer et al., 2010).

A study of such hybridization phenomena in parasites would shed light on epidemiological, ecological and phenotypic advantages eventually acquired by hybrid specimens. Hybridization events in a parasite species could have an impact on its adaptive radiation and diversification. Among these, a differential potential transmission of hybrids to a new host, the different adaptation to an intermediate host, or the colonization of a wider geographical range. These advantages could benefit hybrid genotypes with respect to the parental taxa, resulting in a possible adaptation to new or variation of biotic and abiotic factors (King et al., 2015).

The number of hybrid specimens so far detected between sibling species of the *A. simplex* (s. l.) complex species is too limited to make plausible assumptions about underlying ecological aspects. A more complete dataset could help to further elucidate these dynamics, by collecting and analysing the two parasite species in other hosts and from different basins where they geographically overlap.

Table 5
Allele frequencies observed at eleven microsatellite loci of *A. pegreffii* and *A. berlandi* here studied. As regards sex-linked loci (*; i.e. *Anisl 00314*, *Anisl 7*, *Anisl 4*, *Anisl 22*), the most reliable estimate of allele frequencies was calculated according to the sex-linked genetic model estimate, assuming: *(i)* the hemizygosity of the males at that locus in the two *Anisakis* species; *(ii)* their adult female counterparts, as biallelic at the sex-linked loci.

Locus	Alleles	*A. pegreffii*		*A. berlandi*	
		A + L4	L3	A + L4	L3
Anisl 00185	*182*	0.01	–	0.01	–
	185	–	–	0.01	–
	188	0.02	0.05	0.03	0.01
	191	0.06	0.04	0.07	0.15
	194	0.21	0.27	0.16	0.17
	197	0.21	0.26	0.21	0.19
	200	0.17	0.26	0.29	0.21
	203	0.25	0.11	0.10	0.12
	206	0.05	0.01	0.07	0.12
	209	0.02	–	0.03	0.01
	212	–	–	0.02	0.02
*Anisl 00314**	*96*	0.05	–	–	–
	100	0.28	–	1.00	–
	104	0.32	–	–	–
	108	0.25	–	–	–
	112	0.06	–	–	–
	116	0.01	–	–	–
	120	0.02	–	–	–
	124	0.01	–	–	–
Anisl 10,535	*125*	0.01	–	–	–
	128	0.01	0.01	1.00	0.99
	131	0.01	0.01	–	0.01
	134	0.19	0.20	–	–
	137	0.21	0.18	–	–
	140	0.27	0.27	–	–
	143	0.28	0.28	–	–
	146	0.01	0.04	–	–
	149	0.01	–	–	–
	152	–	0.01	–	–
Anisl 05784	*66*	–	0.01	–	–
	69	0.01	–	0.96	0.99
	72	0.01	–	0.04	0.01
	75	0.01	0.05	–	–
	78	0.05	0.02	–	–
	81	0.03	0.04	–	–
	84	0.07	0.09	–	–
	87	0.25	0.23	–	–
	90	0.32	0.40	–	–
	93	0.18	0.12	–	–
	96	0.05	0.04	–	–
	99	0.01	–	–	–
	102	0.01	–	–	–
Anisl 08059	*82*	0.02	0.01	0.01	–
	86	0.19	0.19	0.77	0.85
	90	0.05	0.06	0.20	0.14
	94	0.12	0.19	0.02	0.01
	98	0.17	0.16	–	–
	102	0.20	0.16	–	–
	106	0.11	0.08	–	–
	110	0.04	0.05	–	–
	114	0.05	0.02	–	–
	118	0.01	0.01	–	–
	122	0.01	0.05	–	–
	126	0.01	0.02	–	–
	130	0.01	–	–	–
	134	0.01	–	–	–
Anisl 00875	*142*	–	–	–	0.01
	145	0.02	–	–	0.01
	148	0.02	–	0.96	0.94
	151	0.04	0.08	0.03	0.04
	154	0.03	0.04	0.01	–
	157	0.68	0.76	–	–
	160	0.15	0.08	–	–
	163	0.05	0.03	–	–
	166	0.01	–	–	–
	169	–	0.01	–	–
*Anisl 7**	*216*	0.02	–	–	–

(continued on next page)

Table 5 (*continued*)

Locus	Alleles	*A. pegreffii*		*A. berlandi*	
		A + L4	L3	A + L4	L3
	219	0.07	–	–	–
	222	0.84	–	–	–
	225	0.07	–	0.01	–
	228	–	–	0.01	–
	237		–	0.01	–
	243	–	–	0.01	–
	252	–	–	0.01	–
	255		–	0.01	–
	258	–	–	0.06	–
	261	–	–	0.07	–
	264	–	–	0.05	–
	267	–	–	0.19	–
	270	–	–	0.19	–
	273	–	–	0.14	–
	276	–	–	0.04	–
	279	–	–	0.04	–
	282	–	–	0.08	–
	285	–	–	0.01	–
	288	–	–	0.02	–
	291	–	–	0.01	–
	294	–	–	0.02	–
	297	–	–	0.01	–
	306	–	–	0.01	–
*Anisl 4**	*127*	0.04	–	0.13	–
	130	0.06	–	0.02	–
	133	0.31	–	0.02	–
	136	0.12	–	–	–
	139	0.16	–	0.37	–
	142	0.16	–	0.08	–
	145	0.07	–	0.04	–
	148	0.02	–	–	–
	151	0.02	–	0.02	–
	154	0.02	–	0.04	–
	157	0.01	–	0.05	–
	160	–	–	0.13	–
	163	–	–	0.04	–
	166	–	–	0.02	–
	169	–	–	0.02	–
	172	-	-	0.02	-
	175	0.01	-	-	-
*Anisl 22**	*179*	0.02	–	1.00	–
	182	0.04	–	–	–
	185	0.45	–	–	–
	188	0.40	–	–	–
	191	0.06	–	–	–
	194	0.01	–	–	–
	197	0.01	–	–	–
	200	0.01	–	–	–
Anisl 15	*231*	1.00	1.00	–	–
	235	–	–	1.00	1.00
Anisl 2	*245*	–	–	1.00	1.00
	248	1.00	1.00	–	–

The mtDNA *cox*2 gene locus has proved its utility in the identification of all the species so far included in the genus *Anisakis* (Valentini et al., 2006), and in the phylogenetic constructions in concatenated analysis based on this locus (Mattiucci et al., 2018b). In addition, this molecular marker can add insight at the intraspecific level in the species of the *A. simplex* (s. l.) complex, concerning knowledge on possible population structuring and phylogeography (Baldwin et al., 2011; Mattiucci et al., 2018b), as well as in the estimating the genetic variability (Mattiucci et al., 2018a). However, its use as single marker in the identification of specimens of the species of the *A. simplex* (s. l.) complex, especially from sympatric areas, may be misleading in the discrimination of specimens between parental taxa and hybrid categories, also because of the lack of information, so far, of recombination in the mitochondrial genome of these parasites. For instance, the detection of those three F1 hybrid genotypes would have been misidentified as *A. berlandi* if mtDNA *cox*2 locus would have been the only locus tested in the present work (Table 2).

4.2. SSR sex-linked loci and their utility in gender determination of larval specimens

Sex-linked microsatellite loci are genes located on the X-chromosome. In the present work, four sex-linked loci (i.e., *Anisl 00314*, *Anisl 7*, *Anisl 4* and *Anisl 22*) have been discovered on the total panel of eleven DNA microsatellite markers so far studied in *A. pegreffii* and *A. berlandi*. As stated in the previous paragraph, the loci *Anisl 7* and *Anisl 22* are partially diagnostic between the two species. Therefore, when male and female specimens are available, it is advisable to verify if sex-linkage occurs in the SSR loci. To isolate a putative sex-linked locus, it is necessary to separate, on the basis of sex, the genotypes of the nematode sample. Where dealing with a sex-linked SSR locus, not all the genotypes observed can be likely used in the Bayesian clustering genotyping inference by STRUCTURE, irrespective of the sex identification. In fact, if the male specimens are homozygous at a considered microsatellite locus, it means that they are not “real” homozygous, but rather hemizygous. Having the chromosome(s) inherited from the mother only, without the chromosomal paternal counterpart, they possess just one copy of that gene. Therefore, taking into account that males are hemizygous (that is monoallelic), while females are heterozygous (that is biallelic), allele frequencies and allele frequencies-based analyses can be then calculated, as it has been carried out in the present study.

While the proportion of sex-linked microsatellites on the whole genome of the species of *A. simplex* (s. l.) was roughly estimated around the 25% in our previous SSRs analysis (Mattiucci et al., 2019), the present study updates this percentage, that should be considered up to 33%, due to the scoring of further DNA microsatellite loci. This value represents a higher percentage in comparison to the 20% that Criscione et al. (2007a) supposed for ascarids (such as *Ascaris lumbricoides*). To date, karyotypes of anisakid nematodes were not determined. In ascaridoid nematodes, males are XO, while females are XX. Maternal gametes always contain an X chromosome, so the sex of the “offspring” specimens depends on whether a sex chromosome occurs in the male gamete. Its sperm normally contains either X chromosome or no sex chromosome at all. In the evolution of sex chromosomes of parasitic nematodes, it is retained that the XO sex chromosome system is the ancestral state in nematodes, while the XY is the derived state (Foster et al., 2020). Some nematodes such as the non-parasitic *Caenorhabditis elegans* have a XO sex-determination system, while others, such as the filarial parasite *Brugia malayi*, have an XY mechanism (Foster et al., 2020). However, the only reference available for ascaridoid nematodes regards *Ascaris suum,* which shows 19 autosomes and 5× chromosomes (2n = 38A + 10× in females, 38A + 5× in males) (Müller and Tobler, 2000). Future cytological work would shed light on the sex chromosomes of anisakid parasites and their evolutionary significance.

Besides being important for population genetics studies, microsatellites can be also a useful and powerful tool to distinguish females from males in organisms with X0 sex determination (Harvey and Viney, 2001). The sex-associated SSR markers so far disclosed in the species of *A. simplex* (s. l.) complex and in the present study, can be of help in revealing an alternative mode of sex identification in their developmental stages (Mattiucci et al., 2019). In fact, if male hemizygosity is assumed, sex-linked loci could likely allow identification of any female at any life-history stage (even as L3 larva or L4 immature worm), when a larval individual shows a heterozygote genotype at one or more of those loci. According to this assumption, for instance, the heterozygote genotypes found, at least, in one of the four sex-linked loci, would allow the identification as female $N = 55$ of L3 and L4 stages of *A. pegreffii* analysed. Similarly*,* $N = 52$ of L3 and L4 larval stages of *A. berlandi,* being heterozygotes at the loci *Anisl 7* and/or *Anisl 4* (the other two loci *Anisl 00314* and *Anisl 22* are monomorphic in this species), can be assumed to be female. However, the occurrence of homozygote genotypes in females could also occur. Thus, in future studies, estimating the statistical occurrence of homozygous genotypes in large number of adult females on as many as possible sex-linked loci, a high probability of

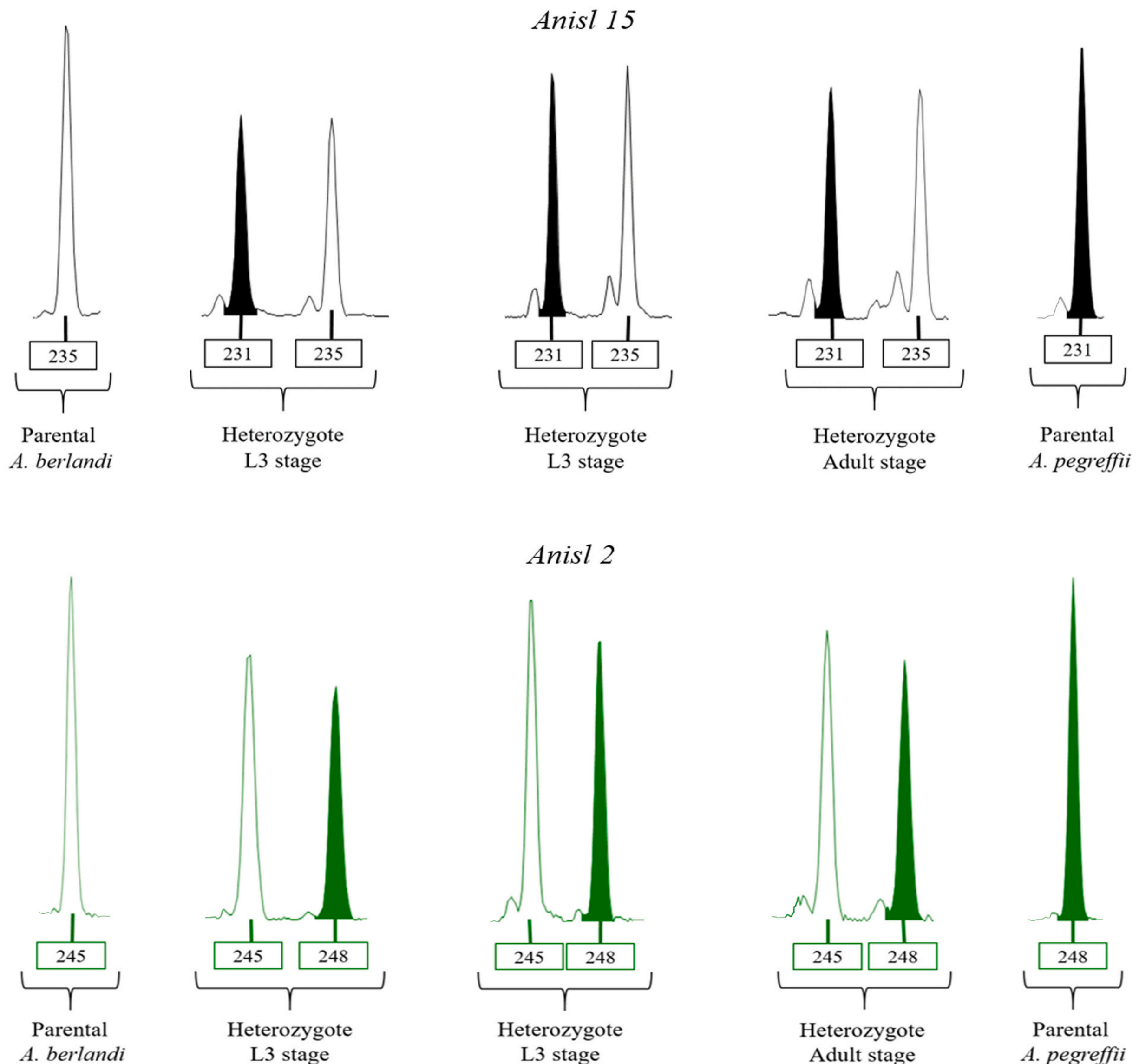

Fig. 3. Electropherograms and observed genotypes of pure parental *A. berlandi* (on the left), F1 hybrids (in the middle) and pure parental *A. pegreffii* (on the right) at the diagnostic loci (100%) between the two *Anisakis* species (i.e., *Anisl 15* and *Anisl 2);* Discriminant alleles for *A. berlandi* are given as empty peaks; discriminant alleles for *A. pegreffii* are shown as full peaks.

correct sex identification of third and fourth larval stage specimens of these parasite species could be reached.

4.3. Genetic differentiation and variability of A. pegreffii and A. berlandi

The genetic/molecular results obtained further confirmed that the two studied taxa are genetically distinct in both nuclear and mitochondrial markers. The multilocus genotyping approach (STRUCTURE) based on nuclear genes of the two parasite taxa has demonstrated that the analysed metapopulations, despite the wide host range, were strongly associated with two distinct panmictic units, corresponding, respectively, to the two species *A. pegreffii* and *A. berlandi*. The result was also corroborated by the pairwise F_{ST} estimates found at the interspecific level, at both nuclear (as based on SSR loci) and mitochondrial (mtDNA *cox*2) gene level.

Whereas, a high genetic homogeneity across sample populations of the two species *A. pegreffii* and *A. berlandi* collected from different fish and cetacean host species was observed occurring in the contact zone of New Zealand waters. The observed values are in agreement with those previously recorded, among populations of the same species, in samples from other geographic areas, also based on DNA microsatellite loci (Mattiucci et al., 2019) and mtDNA *cox2* sequences analysis (Mattiucci et al., 2014).

At the intrapopulation level, previous scoring of SSR loci based on adult populations of *A. berlandi* have detected F_{IT} values higher than zero (Bello et al., 2020), likely suggesting a possible certain subdivision between populations of this parasite species. Whereas, in the present study, the additional scoring of a larger number of specimens, including larval forms from several fish species, has shown a not significant $F_{IT,}$ and F_{IS} values, being those very close to zero (Table 6). This result provides no evidence for deviation from random mating within infrapopulations of *A. berlandi* from this area of the Pacific, i.e., the existing of panmixia. The partitioning of genetic differentiation between and within the populations of *A. pegreffii* studied at the SSRs loci, have shown that most of the diversity is within populations, being also the F_{ST} among populations very close to zero (Table 6). This again implies the existence of a high level of gene flow between the populations of *A. pegreffii* inhabiting New Zealand waters, despite the high genetic polymorphism presently disclosed at 11 DNA microsatellite loci. At the intraspecific level, a moderate genetic differentiation (on average, $F_{ST} = 0.060$) estimated on 7

Fig. 4. Percentage contribution (Q value) of *A. pegreffii* and *A. berlandi* species to the multilocus genotype of each studied individual (barplot), estimated at five SSR loci (i.e., *Anisl 10,535, Anisl 05784, Anisl 00875, Anisl 15* and *Anisl 2*) and at the nuclear gene *nas10*, by using STRUCTURE with k = 2 (A), and probability of assignment to a particular hybrid class estimated using NEWHYBRIDS (B) (Anderson and Thompson, 2002).

Table 6
Analysis of molecular variance (AMOVA) between and within adult and L4 stages group vs L3 stages group, of the two species *A. pegreffii* (a) and *A. berlandi* (b), using genetic data sets from seven SSRs loci (*Anisl 00185, Anisl 10535, Anisl 05784, Anisl 08059, Anisl 00875, Anisl 15* and *Anisl 2*). The *p* values to the fixation indices are reported as ****p* < 0.001; * *p* < 0.05;. n.s., not significant.

a.Anisakis pegreffii					
Source of variation	d.f.	Sum of squares	Variance components	% of variation	Fixation indices
Among populations	1	2.413	0.004	0.22	*Fst* = 0.002 n.s.
Among individuals within populations	185	356.785	0.075	4.05	*FIS* = 0.040*
Within individuals	187	332.500	1.778	95.73	*FIT* = 0.043*
Total	373	691.698	1.857		

b. *Anisakis berlandi*					
Source of variation	d.f.	Sum of squares	Variance components	% of variation	Fixation indices
Among populations	1	0.889	0.002	0.28	*Fst* = 0.003 n.s.
Among individuals within populations	131	86.341	0.016	2.42	*FIS* = 0.024 n.s.
Within individuals	133	83.500	0.628	97.30	*FIT* = 0.027 n.s.
Total	265	170.729	0.645		

SSR loci, has been disclosed between the Boreal populations of *A. pegreffii* from the Mediterranean Sea, and their conspecifics from the New Zealand southern Pacific and Argentine Southern Atlantic (Mattiucci et al., 2019). The parasite population genetic substructuring would be also driven by the population genetic structure of its definitive hosts (Esch and Fernández, 1993). In the present case, the population structure of some cetacean species, from different geographical areas would also be responsible for maintaining such level of genetic substructuring of their endoparasites (i.e., *A. pegreffii*) in Boreal (i.e., Mediterranean Sea) and Austral (i.e., New Zealand) waters. Indeed, for instance, the genetic diversity and population genetic structure of the bottlenose dolphin *Tursiops truncatus* identify its New Zealand population as the most divergent from populations of the Atlantic and Mediterranean waters. Despite this, *T. truncatus* maintains high level of gene flow over long distances (Tezanos-Pinto et al., 2009). This finding seems to mirror the population genetic substructuring so far detected in *A. pegreffii* from different geographical areas included in its range of distribution, as the possible effect of host-parasite relationship. The possible role of biotic factors, in terms of host population density, vagility, and genetic population structure, has been indeed suggested to be a driver in the epidemiology and the genetic structure of the parasites species included in the *A. simplex* (s. l.) complex (Mattiucci et al., 2018a).

Despite the presence of larval and adults of *A. berlandi* from fish and cetacean hosts has been recorded in some areas of the Southern region and, recently, also from the Argentine coast (Irigoitia et al., 2018, 2021), data on its population genetic structure investigated by DNA microsatellite markers and mtDNA *cox*2 are yet scanty. This prevents meaningful discussion about possible spatial genetic diversity between different southern populations of *A. berlandi*. Future analysis of nuclear and mitochondrial gene distributions in populations of the two parasite species from different Boreal and Southern Ocean waters will help provide a worldwide perspective on their population structure and genetic diversity.

Multi-host ascaridoid populations are expected to maintain higher levels of genetic polymorphism than parasites with a narrow host range, because they are unlikely to experience extreme population number fluctuations (Bullini et al., 1986; Mattiucci and Nascetti, 2007, 2008). The results obtained in the present study agree with this prediction, as the polymorphism detected was high at all microsatellite loci, in the

scored populations of *A. pegreffii* (on average, $He = 0.56$); a lower value was observed in *A. berlandi* (on average, $He = 0.28$). A high genetic polymorphism was previously found at other nuclear loci (allozymes) in the populations of the two parasite species from New Zealand waters, with average values of $He = 0.11$ in *A. pegreffii,* and $He = 0.24$ in *A. berlandi* (Mattiucci et al., 1997). High genetic variability has been generally reported in species of the *A. simplex* (s. l.) complex, also inferred from other nuclear gene loci, such as allozymes (Mattiucci and Nascetti, 2008). This finding could be ascribed to the large effective population size of anisakid species generally observed in both intermediate/paratenic (fish) and definitive (cetaceans) hosts. Furthermore, a high level of gene flow exists between conspecific populations of these parasites, maintained by the high vagility of many of their hosts, carrying the parasites over large migration routes, across distant oceanic basins. In the present study, parasitic infection levels exhibited by the two parasite species in different fish species are not given. Additional genetic studies and data on the parasite population density of *A. pegreffii* and *A. berlandi* from different geographic areas of the Southern Region are required, in order to investigate spatial genetic structure of the two parasite species, their larval and adult abundance, their effective population sizes and genetic variability.

5. Concluding remarks

This is the first study employing a wide-genotyping approach to identify adults and larvae of two sibling species of the *A. simplex* (s. l.) complex originating from New Zealand waters. Several *A. simplex* (s. l.) nuclear gene loci, discovered recently and also in this work, have a clear diagnostic capacity to identify *A. pegreffii* and *A. berlandi.* Markers based on these loci have allowed genetic data collection, which elaborated by updated robust Bayesian methodologies, have dramatically raised the possibility of distinguishing parental and hybrid genotypes, and genetically characterising populations of these nematodes. Indeed, despite the fact that DNA microsatellite loci are considered as markers under selection, the scored SSR loci could be used as markers of neutral genetic structure of the two parasite species. All the tested loci have indeed given a similar signal and behave similarly in the analysis. Thus, it appears that these SSR loci reflect the genetic structure of the regions in the genome of those parasite species that are not likely involved in the host–parasite interaction, i.e., regions of the genome that are not under selection.

It has become clear that without a wider investigation of the genetic architecture of these anisakid parasites, based on several nuclear genes, misinterpretation of naturally occurring hybridization and/or introgression events can occur. Indeed, hybridization does not necessarily generate permanent genetic exchange between the taxa; it could be a transient phenomenon. When, on the contrary, interspecific mating events occur between hybrids and their parental species or between hybrid individuals, both events resulting in backcross individuals, a more permanent introgression phenomenon would exist, which could also represent an adaptive trait from one species into the other. However, the current ambiguity surrounding the distinction between hybridization and introgression in the species of *A. simplex* (s. l.) complex, such as for instance, between *A. simplex* (s. s.) and *A. pegreffii,* is mainly due to a tendency to investigate a single locus when, instead, a multi-loci or whole-genome approach would be required.

In the present study, diagnostic genotyping at other nuclear markers, other than those inferred from SSR loci, such as those gathered from metallopeptidase *nas10* nDNA, will help clarify the affinities and genetic structure of anisakid parasites and their populations in sympatric areas. The combined nuclear data sets have allowed the successful detection of current hybridisation between *A. pegreffii* x *A. berlandi*. However, investigations at other nuclear loci, even starting from an available draft genome of *A. simplex* (s. l.) (Łopieńska-Biernat et al., 2019), should be pursued to disclose further diagnostic SNPs at certain gene loci, to aid in differentiating and mapping the hybridization/introgression events between the three species of the *A. simplex* (s. l.) complex in their contact areas. This will also add insights into the phenotypic and functional implications of naturally occurring hybrids between the three taxa.

In addition, future studies employing as many as possible sex-linked loci would be also aimed for sex identification of third and fourth larval stage specimens of these worms. Sex determination in these parasites will facilitate further investigations, such as the significance of relative proportions of adult males/females in definitive hosts in comparison with larval stages occurring in intermediate/paratenic hosts, differential sites of infection in fish hosts, and the effect of differential gene expression levels between male and female specimens. In the last concerns, for instance, differential gene transcriptions between L3 and L4 in *A. pegreffii* and *A. simplex* (s. s.) (Kim et al., 2018) has been demonstrated, as well as the existence of differential gene expression of adaptive genes in those parasite species infecting fish host tissues (Palomba et al., 2020a).

In conclusion, the new and emerging wide-genotyping approach in the species of the *A. simplex* (s. l.) complex could provide information also in their phenotypic and ecological traits, which, in turn, would have also epidemiological and pathological implications. These last aspects acquire human health importance because, while currently *A. pegreffii* and *A. simplex* (s. s.) are known as the etiological agents of human anisakiasis, the zoonotic role of *A. berlandi* is so far unknown (Mattiucci et al., 2018a).

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Authors contribution

Conceptualization, SM, GN, EB, MaP; methodology, EB, MaP, MiP; material sampling: SCW; writing-original draft preparation, SM, EB, MaP, PC, SCW; writing-review and editing, SM, PC, SCW; supervision, SM, GN. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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