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SSR markers as a powerful tool for genetics investigation: *Anisakis simplex* (s. l.) complex (Nematoda: Anisakidae) as a case study

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1. INTRODUCTION

1.1 BACKGROUND

Population genetics is the study of genetic variation within populations. This discipline involves the examination and the modelling of changes in the frequencies of genes and alleles in populations over space and time, investigating the evolutionary factors that explain this variation. Its foundation is the Hardy - Weinberg law, which is maintained as long as population size is large, mating is at random, and mutation, selection and migration are negligible; if those conditions do not occur, allele frequencies and genotype frequencies may change from one generation to the next.

Many of the genes found within a population are polymorphic, which is they occur in a number of different forms (alleles). Mathematical models are used to investigate and predict the occurrence of specific alleles or combinations of alleles in populations, based on developments in the molecular understanding of genetics, Mendel's laws of inheritance and modern evolutionary theory.

The main tools to study population genetics are molecular-genetic markers, any trait used as a marker of genetic variation within and among individuals and taxa. Considered traits include phenotypic traits (i.e. eye colour), protein products (i.e. allozymes, albumin), and segments of DNA (mtDNA, chDNA and nDNA genes).

By definition, a good genetic marker should generally be characterized by some features, such as: (i) Mendelian inheritance, (ii) polymorphic character, (iii) stability, (iv) codominance, (v) widespread occurrence in the genome and (vi) repeatability.

Genetic markers are widely used for identification and molecular dissection of species complexes in animals, including parasites (Anderson *et al.*, 2001).

In the past, the morphological features resulted to be the only tools to distinguish different species of parasites belonging to a same complex or a same genus. However, since the parasite animals underwent (and they undergo to this day) processes of parallel evolution, the taxonomic classification based on only morphological characters turned out to be not enough adequate. Parallel evolution is the independent evolution of similar traits, starting from a similar ancestral condition. Frequently, this is the situation in more closely related lineages, where several species respond to similar challenges in a similar way.

As regards parasite animals, although they can infect different kind of hosts, they do not need too differentiated phenotypical traits to survive, and consequently different taxa appear very similar to each other from a morphological point of view, especially as regards a same genus or a same species complex. Thus, molecular-genetic approaches were resulted to be essential for discriminating a taxon from another one, and for establishing the genetic relationships between different species.

Parasite animals belonging to the phylum Nematoda are ubiquitous and show a huge diversity of life strategies, with direct or indirect life cycle, displaying different reproductive modalities and degrees of host specialisation (Gilabert and Wasmuth, 2013).

Genus *Anisakis* (Dujardin, 1845) belongs to the phylum Nematoda and to the order Ascaridida. *Anisakis* are marine endoparasites that display a complex life cycle, comprising different kinds of hosts through the marine food web. To go into detail, adult specimens belonging to this genus mate and reproduce in the digestive system of different species of cetaceans (especially Odontocetes), and release their eggs in the marine environment through the defecation of their definitive hosts. At the eggs hatching, free-swimming unsheathed

larvae are released and can undergo one or two moults before being ingested by small invertebrates, which act as intermediate hosts. In those small crustaceans (planktonic or semiplanktonic), larvae occur at the third larval stage (L3) and, through predation phenomena, they pass into one or more paratenic hosts, as fish or cephalopods species, without undergoing any moult. Finally, the third larval stages reach their definitive host, where they undergo the final moult in the fourth larval stage (L4) and then mature in the adult and sexually reproductive stage (see Fig. 1 for details).

In the life cycle of *Anisakis* species, humans can act as the accidental hosts of these parasites, consuming raw, smoked, marinated salted or undercooked fish and squids infected by larval stages. The zoonosis caused by the infection of *Anisakis* larvae is known as "anisakiasis" and represents a global emerging problem for the human health (Chai *et al*., 2005; Buchmann and Mehrdana, 2016; Guardone *et al*., 2018; Mattiucci *et al*., 2018a) (Fig. 1).

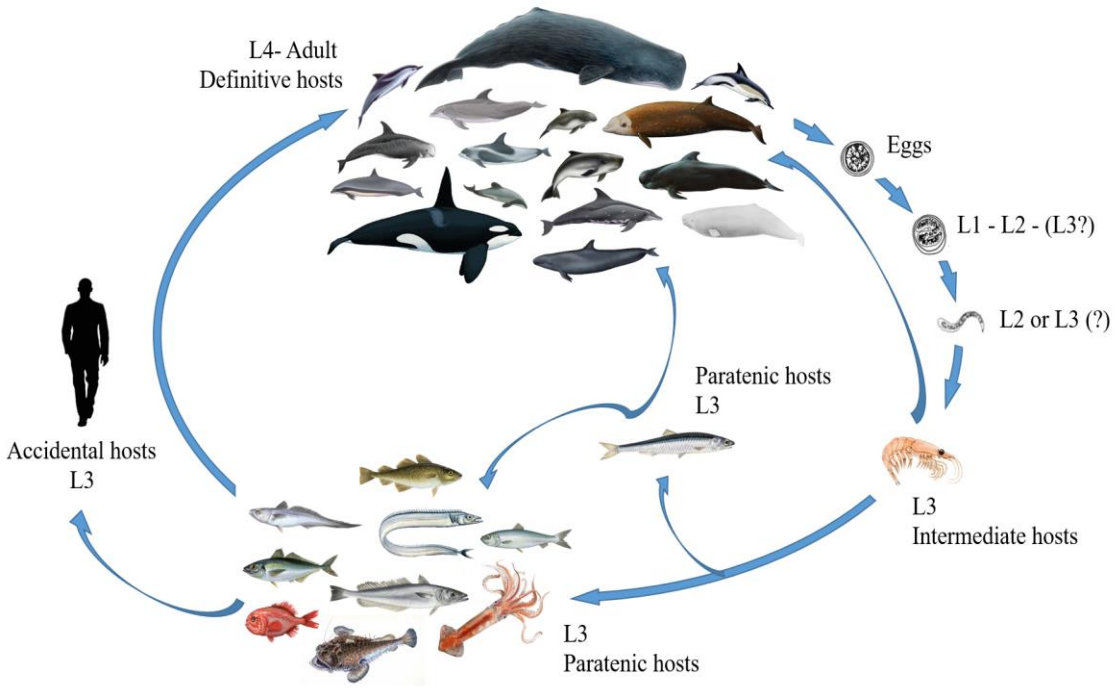

Figure 1: Life cycle of marine endoparasites belonging to genus *Anisakis*. Original figure.

To date, nine species were described and identified for the genus *Anisakis*: *A. simplex* (s. s.), *A. pegreffii*, *A. berlandi*, *A. typica*, *A. nascettii*, *A. ziphidarum*, *A. physeteris*, *A. paggiae* and *A. brevispiculata*. All those species were characterized by distinct diagnostic genetic markers, possess distinct gene pools, and are reproductively isolated (Mattiucci *et al.*, 2018a).

Other two taxa, i. e. *Anisakis* sp. 1 *(*closely related to *A. typica)* and *Anisakis* sp. 2 *(*closely related to *A. physeteris)* were reported, but more genetic/molecular analyses are needed to clarify the taxonomic status of those parasites (Mattiucci *et al*., 2018a).

Phylogenetic analyses on nuclear and mitochondrial genes showed the existence of four distinct clades in this genus of marine endoparasites (Valentini *et al*., 2006; Mattiucci and Nascetti, 2008; Cavallero *et al*., 2011; Mattiucci *et al*., 2014a), and this subdivision was further confirmed by some morphological features detected in the species clustering in those clades (Mattiucci *et al*., 2018a).

Clade I includes three sibling species, i.e. *A. simplex* (s. s.), *A. pegreffii* and *A. berlandi*, parasite worms that colonize preferably marine mammals belonging to the Superfamily Delphinoidea (i.e Delphinidae, Phocoenidae, Monodontidae). They share some common morphological characteristics at the adult stage: (i) the ventriculus is longer than broad and often sigmoid in shape, and (ii) male spicules are long, thin, and unequal in length (Mattiucci *et al.*, 2014a).

Clade II includes two sibling species, i.e. *A. ziphidarum* and *A. nascettii*, that preferably parasitize beaked whales belonging to genera *Ziphius* and *Mesoplodon*. *A. ziphidarum* mainly colonizes the first aforementioned genus, while *A. nascettii* mainly infects the latter one. At the adult stage, those species share some common morphological features,

which is (i) a long ventriculus, not sigmoid in shape, and (ii) long, thin, and equal in length male spicules (Paggi *et al.,* 1998; Mattiucci *et al*., 2009).

Clade III consists of three sibling species, i.e. *A. physeteris*, *A. brevispiculata* and *A. paggiae*, preferably infecting sperm whales. The first nematode mainly parasitizes the *Physeter macrocephalus,* while the second and the third nematodes mainly infect pigmy and dwarf sperm whales, i.e. *Kogia breviceps* and *K. sima*. Their characteristic morphological traits at the adult stage are (i) the short, never sigmoid and broader than long ventriculus, and (ii) short, stout, and of similar length male spicules (Mattiucci *et al*., 2005).

Clade IV includes *A. typica* species only; in fact, from concatenated phylogenetic trees obtained from the combined nuclear and mitochondrial sequences identified, this species resulted to be as a separate lineage with respect to the other species. It preferably infects cetaceans belonging to the Superfamily Delphinoidea living in tropical waters. Adults of this species show (i) a long ventriculus, (ii) long, thin and very marked unequal in length (mean ratio 1:3) male spicules; (iii) lips with an anterior bilobed dorsal lip; (iv) paracloacal papillae not double (Davey, 1971; Mattiucci *et al*., 2018a).

As regards larval stages, clade I, II and IV share the same Type of larva (i. e. Type I), which is characterized by the presence of a mucron at the tail end and a long ventriculus (sensu Berland, 1961). On the contrary, larval stages of clade III can be distinguished in Type II (*A. physeteris*), Type III (*A. brevispiculata*) and Type IV (*A. paggiae*) (sensu Berland, 1961; sensu Shiraki, 1974). Those types of larval stages are characterized by a short ventriculus and a tail without mucron but morphologically different, being conical and tapering in Type II, rounded in Type III, and short and pointed in Type IV (Shiraki, 1974).

Phylogenetic relationships and morphological features of clades belonging to genus *Anisakis* are summarised in Fig. 2, from Mattiucci *et al*., 2018a.

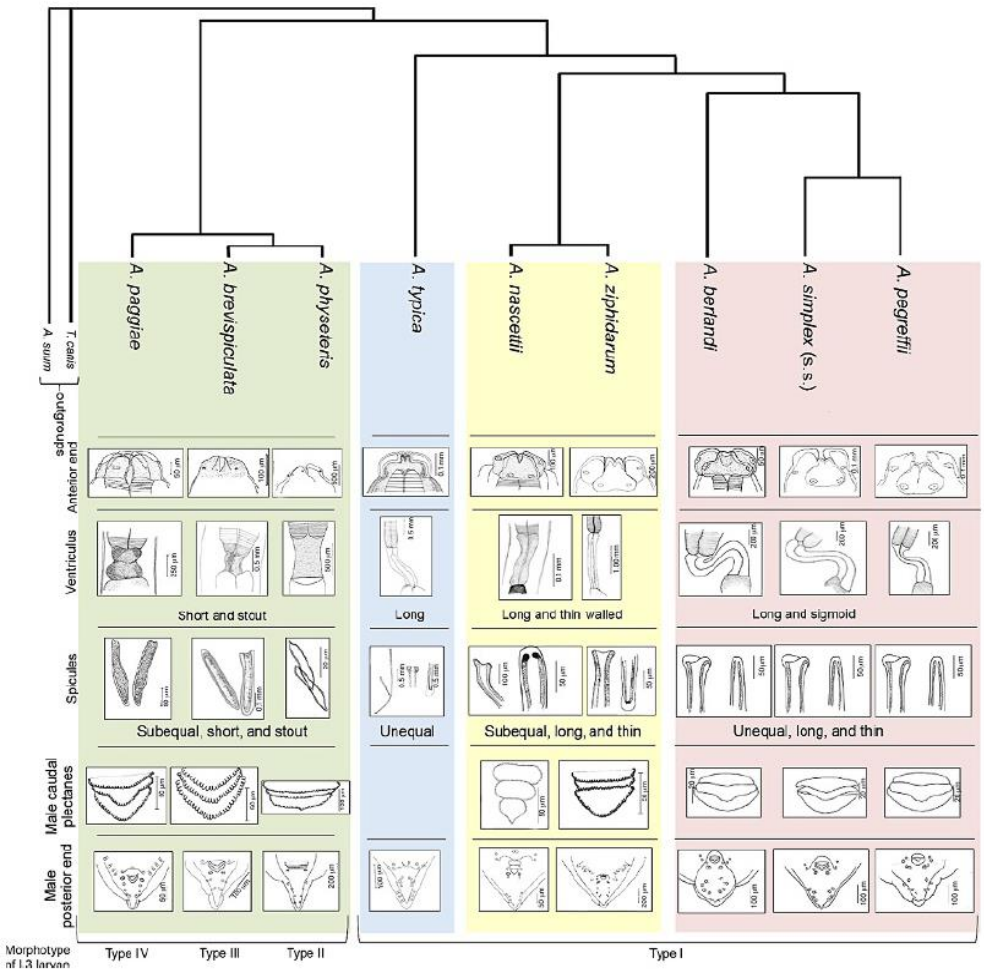

Figure 2. Some morphological/morphometric diagnostic features among the genetically characterized species of *Anisakis*, mapped into their phylogenetic tree. Distinctive characters selected are: male cephalic end, ventriculus length and shape, spicules length and shape, ratio between the right and left spicule lengths (R/L), male caudal end and arrangement of caudal papillae, male caudal plates, and larval (L3 stage) morphotype (from Mattiucci *et al*., 2018a)

Species belonging to genus *Anisakis* show a wide range of distribution, which includes northern and southern waters of the world (see Mattiucci *et al*., 2018a, for a review).

Of the nine species belonging to the genus *Anisakis*, the most investigated are the three sibling species of the *A. simplex* (s. l.) complex [i.e. *A. pegreffii*, *A. simplex* (s. s.) and *A. berlandi*], in particular *A. pegreffii* and *A. simplex* (s. s.). In fact, those two species are reported as the main causes of anisakiasis in humans, as L3 larval stages (Mattiucci *et al*.,

2018a), and thus studying them from both an ecological and epidemiological point of view results to be very important (Mattiucci *et al.*, 2018a).

A. simplex (s. l.) complex is supported by concatenated inference, obtained from both MP analysis based on combined mitochondrial sequences dataset and combining both mitochondrial and nuclear sequence data (Mattiucci *et al*., 2014a; Mattiucci *et al*., 2018a). They gave identical topologies in supporting the existence of the three species as distinct phylogenetic lineages (Mattiucci *et al*., 2018a).

A. simplex (s. s.) (=*A. simplex* B, Nascetti *et al*., 1986) shows a distribution that ranges approximately from 35°N to the Artic Seas, circumpolarly. Moreover, this taxon occurs along the Pacific coasts of Japan and along the North American coasts (see Mattiucci *et al*., 2018a, for a review). *A. simplex* (s. s.) is not very common in Southern waters: this species was reported at larval stages only in Alboran Sea and rarely in Mediterranean Sea, maybe because of the migratory routes of paratenic and definitive hosts, as *Merluccius merluccius*, *Scomber scombrus* and *Thunnus thynnus* (Levsen *et al*., 2017a; Mladineo *et al*., 2017a). As regards cetaceans, in the Mediterranean Sea only three specimens belonging to this parasite species were recognized from *Tursiops truncatus* and *Stenella coeruleoalba* (Blažeković *et al*., 2015; Mladineo *et al*., 2017a). To date, 11 species belonging to the Superfamily Delphinoidea were reported as definitive hosts of *A. simplex* (s. s.) (i.e. *Delphinapterus leucas, Delphinus delphis, Globicephala melas, G. macrorhynchus, Lagenorhynchus albirostris, Orcinus orca, Phocoena phocoena, Pseudorca crassidens, Stenella coeruleoalba, Steno bredanensis, Tursiops truncatus*); accidental infections were reported in whales as *Balaenoptera acutorostrata* and in sperm whales (i.e. *Physeter macrocephalus, Kogia breviceps*). As regards the paratenic hosts, this parasite species was reported to infect above 50 pelagic, benthopelagic and demersal teleost fish and four squid species (Mattiucci *et al*., 2018a).

A. pegreffii (Campana-Rouget and Biocca, 1955; =*A. simplex* A, Nascetti *et al*., 1986) shows a wide geographical distribution, that includes the whole Mediterranean basin, the Atlantic coast of Iberian peninsula, Taiwan Sea, East China Sea, Korean and Japanese waters, and the Austral regions between 30°S and 60°S (see Mattiucci *et al*., 2018a, for a review). It is a very rare circumstance that *A. pegreffii* occurs in Northern waters: the sporadic identification of this taxon in the Northern Atlantic waters of Norwegian Sea could be due to the observed changing migratory route of the Southern stock of *Scomber scombrus* in the NE Atlantic waters (Levsen *et al*., 2017a,b). *A pegreffii* was reported to infect several pelagic and demersal fish as paratenic hosts, while was recognized in eight species of cetaceans belonging to the Superfamily Delphinoidea (i.e. *Cephalorhynchus hectori, Delphinus delphis, Globicephala melas, Grampus griseus, Lagenodelphis hosei, Phocoena phocoena, Stenella coeruleoalba, Tursiops truncatus*) as definitive hosts. As regards accidental hosts, *A. pegreffii* was reported in *Caperea marginata*, the only whale belonging to the family Neobalenidae, and in *Ziphius cavirostris.*

A. berlandi (=*A. simplex* C, Mattiucci *et al*., 1997, Mattiucci *et al*., 2014a) is discontinuously distributed in the waters of the world. To date, the geographical range of distribution of this taxon includes the Chilean Pacific waters, the South Shetland Islands, the New Zealand waters, the Southern African Atlantic coasts, the Pacific coast of Canada (Vancouver Island) and the Californian coasts (see Mattiucci *et al*., 2018a for a review). To date, *A. berlandi* was reported at larval stage in twelve species of fishes (i.e. *Allocytus niger, Anoplopoma fimbria, Electrona carlsbergi, Etrumeus whiteheadi, Gymnoscopelus nicholsi, Hoplostethus atlanticus, Macruronus novaezelandiae, Parapercis colias, Pseudocyttus maculatus, Pseudophycis bachus, Sebastolobus alascanus, Thyrsites atum*) as paratenic hosts. As regards definitive hosts, this species was reported in three cetaceans belonging to the Superfamily Delphinoidea (i. e. *Globicephala melas, Lissodelphis borealis, Pseudorca*

crassidens), while in *Kogia sima*, *Ziphius cavirostris* and the pinniped *Mirounga leonina* as accidental hosts (Mattiucci and Nascetti, 2008; Mattiucci *et al*., 2017a).

For a more detailed point of view of range of distribution of taxa belonging to *Anisakis simplex* (s. l.) complex see Fig. 3.

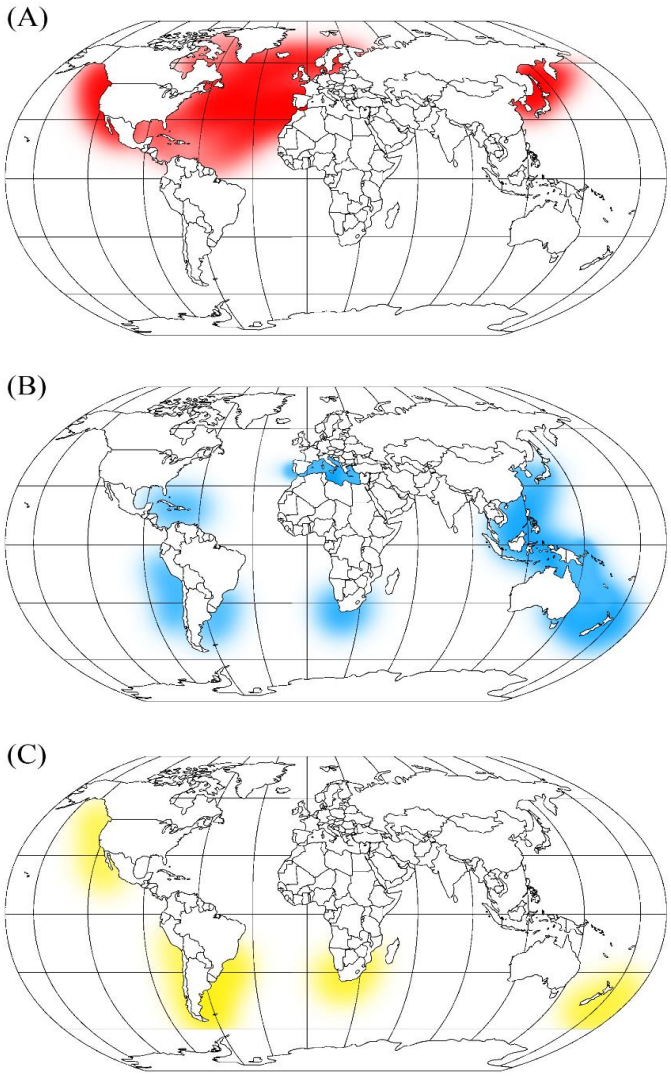

Figure 3: Geographical range of distribution of species belonging to *Anisakis simplex* (s. l.) complex; (A) *A. simplex* (s. s.); (B) *A. pegreffii*; (C) *A. berlandi*. Original figure.

The three sibling species belonging to the *A. simplex* (s. l.) complex were identified first by allozyme markers (Nascetti *et al*., 1986, Mattiucci *et al*., 1997): genotyping approach

from Multilocus Allozyme Electophoresis (MAE) allowed genetically characterizing those species, to estimate their genetic differentiation and their genetic variability. Later, the *A. simplex* (s. l.) complex was studied by mitochondrial and nuclear markers, i.e. mtDNA *cox-2* gene (Valentini *et al*., 2006, Mattiucci *et al*., 2014a), ITS region of rDNA (D'Amelio *et al*., 2000) and EF1 α-1 (nDNA) gene (Mattiucci *et al*., 2016). Combining in a multilocus genotyping approach all these markers (when it is possible) allows us to increase our knowledge about those parasite species. In fact, a multilocus genotyping approach allows: (i) establishing genetic relationships between related taxa; (ii) providing intraspecific and interspecific genetic population analyses; (iii) identifying these parasites at any stage of their life-cycle.

Multilocus genotyping approach is a very powerful tool to study cryptic and sibling species, because it makes possible to disentangle them, with a greater degree of reliability. Discovering and developing new molecular-genetic markers with diagnostic power represent an efficient strategy to investigate complicated phenomena.

Among the contents that can be investigated by a multilocus genotyping approach, phenomena as hybridization and introgression between related species can be considered. Hybridization occurs when two genetically distinct specimens (belonging to different species, genera and even families) can mate and produce offspring. This offspring generally can be infertile: in fact, the homologue chromosomes in the hybrids are composed of one chromosome of a parental species and one chromosome of the other parental species, and thus they do not properly segregate during the meiosis. Nevertheless, sometimes hybrids are fertile (solely because of chromosomal disorder, as polyploidy) and can mate either with one of their parental species (backcrossing or introgression) or with other hybrids.

The role and the extent that hybridization plays in the evolution of parasitic organisms remains insufficiently investigated (Arnold, 2004). Hybridization can have different effects on the species, as introgression of novel genes, divergence via reinforcement, and homogenization across the genomes of interbreeding populations or rapid adaptive diversification (Barton 2001, Olden *et al*., 2004, Seehausen, 2004). Studying these processes is crucial to understand parasite evolution and epidemiology, in particular, when the hybridization can lead to the evolution of more virulent genotypes or showing reduced host specificity (Arnold, 2004).

Parasite species belonging to the *A. simplex* (s. l.) complex share some distribution areas and they were often reported in a condition of syntopy in the same host, both paratenic and definitive one. Syntopy of these species in paratenic hosts is more common in comparison to syntopy in definitive hosts. So far, Iberian Atlantic coasts was reported as the sympatric area for *A. simplex* (s. s.) and *A. pegreffii* adult stages; mixed infection in *Delphinus delphis* was identified by Abollo *et al*., (2003). Adult *A. simplex* (s. s.) and *A. berlandi* specimens were identified in the same definitive host (i. e. *Pseudorca crassidens*) along Vancouver Island (Mattiucci *et al*., 1997). Finally, adult *A. pegreffii* and *A. berlandi* specimens were reported in syntopic infection along the coasts of New Zealand, in *Globicephala melas* (Mattiucci *et al.*, 2014a).

To date, only one case of mixed infection of the three sibling species in a same fish host was reported throughout California Current system (i.e. in *Sardinops sagax*, Baldwin *et al*., 2011), but no syntopic infections of adults in the same definitive host were emerged.

Syntopy of sexually mature specimens can results to be a good condition for hybridization. After all, although two closely related endoparasite species were reported to be perfect biological species (BSC) (Mayr, 1963), as the case of taxa belonging to *A. simplex* (s.

l.) complex, the direct contact between them into a definitive host could contribute occasionally to phenomena of outbreeding.

About *A. simplex* (s. l.) complex, so far some cases of hybridization between *Anisakis simplex* (s. s.) and *A. pegreffii* species were reported, but never between *A. simplex* (s. s.) and *A. berlandi*, or between *A. pegreffii* and *A. berlandi*.

In the first aforementioned case, F1 hybrids between *A. simplex* (s. s.) and its related species *A. pegreffii* were reported in many studies, both in allopatric (Farjallah *et al.*, 2008; Meloni *et al*., 2011; Chaligiannis *et al*., 2012; Cavallero *et al*., 2012, 2014; Pekmezci *et al.*, 2014; Costa *et al*., 2016) and sympatric areas (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). However, it is important to emphasize that those heterozygote genotypes were identified by the employment of only one nuclear marker, such as ITS region of rDNA (D'Amelio *et al*., 2000).

As reported by Anderson *et al.,* 2001, using a single locus marker to study closely related species can results to be "dangerous", especially as regards parasite animals: in fact, the correct way for investigating is multilocus genotyping approach (Anderson *et al.*, 2001).

Therefore, in 2016, Mattiucci *et al*. implemented a multilocus genotyping approach, which allowed proving how employing a single genetic marker could be misleading. In fact, the results of that study proved that the ITS region of rDNA should not be considered as a perfect diagnostic between *A. pegreffii* and *A. simplex* (s. s.) species and their "putative" hybrids. Indeed, one of the two discriminant positions on the genetic sequence of ITS region (i. e. 294 bp, D'Amelio *et al.*, 2000) resulted to be a shared polymorphism between *A. simplex* (s. s.) and *A. pegreffii* species (Mattiucci *et al.*, 2016). Thus, in the light of this, ITS region should not even considered as a powerful tool to identify F1 hybrids, since the shared

polymorphism overestimates them, in both sympatric and allopatric areas (Mattiucci *et al*., 2016). The conclusion about ITS region was reached considering the genotypes obtained at diagnostic nuclear genes, such as allozymes (i.e. *Adk-2, Pep C1*, *Pep C2*, *Sod-1*) and EF1 α-1 gene (Mattiucci *et al*., 2016): in fact, PCR-RFLP analysis of ITS rDNA revealed 30 "putative" hybrids *A. pegreffii* x *A. simplex* (s. s.), both in allopatric areas (i. e. New Zealand, North Sea and Mediterranean Sea) and sympatric areas (i. e. Iberian Atlantic coast and Alboran Sea), but only 11 of them were also confirmed by the multilocus genotyping approach consisted of allozymes and EF1 1-α gene. Thus, 19 putative hybrids at ITS region resulted to be as "pure" specimens at the multilocus genotyping approach consisting of the other five nuclear markers, and such a discordance resulted to be very significant.

Therefore, multilocus genotyping approach allowed identifying "pure" parental genotypes belonging to *A. simplex* (s. s.) and *A. pegreffii* species, both in allopatric and sympatric areas. Furthermore, it allowed to indentify with greater reliability "putative" hybrid specimens, that showed heterozygote genotypes at all the aforementioned diagnostic nuclear loci (i.e. *Adk-2, Pep C1*, *Pep C2*, *Sod-1* and EF1 1-α) (Mattiucci *et al*., 2016). In that study no introgressive hybridization was detected, supported by the evidence that no backcrossing with the two parental species was observed (Mattiucci *et al*., 2016, 2018a).

As stated above, *A. simplex* (s. s.) and *A. pegreffii* species were reported in many cases living in syntopy in the same definitive host. In light of this, a possible explanation for the events of hybridization between *A. simplex* (s. s.) and *A. pegreffii* species was the general higher number of adult females belonging to *A. simplex* (s. s.) species in comparison to the adult females belonging to the related species in the definitive host. Therefore, the outbreeding between *A. simplex* (s. s.) females and *A. pegreffii* males could be more probable (Mattiucci *et al*., 2016). This hypothesis was formulated considering the results of the

identification by mtDNA *cox-2* gene, where F1 hybrids specimens showed the maternal lineage *A. simplex* (s. s.) in the majority of the circumstances. Thus, the absence of conspecific pairing partners and mating stimuli for females of rarer species may be important factors in increasing the likelihood of interspecific current hybridization (Mattiucci *et al*., 2016).

However, despite that recent and detailed study about hybridization phenomenon in *A. simplex* (s. l.) complex, to date little has known about it. Thus, in order to contribute to the knowledge of this content, new molecular and diagnostic genetic markers should be developed/studied/analysed to increase the investigative power of multilocus genotyping approach: in this regard, one of the more "tempting" options were microsatellite loci.

Microsatellites, also known as simple sequence repeats (SSRs), or short tandem repeats (STRs), are tandemly repeated motifs of 1-6 bases found in all prokaryiotic and eukaryotic genomes (Zane *et al.,* 2002). The repetitive structure of microsatellites makes them prone to change of length, undergoing mutations due (i) to errors during DNA replication (strand slippage or replication slippage), (ii) to strand misalignment/slippage during recombination, and, in rare circumstances, (iii) to unequal crossover between misaligned DNA duplexes (homologous chromosomes or sister chromatids) (Jentzsch *et al*., 2013). Thus, due to the three aforementioned molecular processes, the mutation rate of microsatellites results higher than those of point mutations (from 10^{-6} to 10^{-2} events per locus per generation) (Bhargava & Fuentes, 2009). The number of repeat units involved in a mutation can vary, but generally, a single repeat is added or lost per mutational event (Jentzsch *et al*., 2013). Accordingly, SSRs are typically highly polymorphic and different individuals show variation, manifested as repeats number differences (Guichoux *et al.,* 2011). Thanks to their high mutation rate, their codominant character and the relative ease to cross-amplify between

closely related species, microsatellite loci have emerged as one of the most popular choice *(i)* to study contemporary migration rate, *(ii)* to distinguish relatively high rates of migration from panmixia, *(iii)* for fingerprinting, *(iv)* parentage analysis, *(v)* genetic mapping, and *(vi)* genetic structure analyses (Selkoe & Toonen, 2006; Guichoux *et al.*, 2011).

Microsatellites are PCR-based markers and another their important edge concerns the chance to amplify them successfully from poor-quality or low quantities of DNA: that makes them as useful markers to analyse ancient DNA or museum samples (Hodel *et al.*, 2016). Moreover, this advantage results to be very important when the most probable way to obtain samples is the conservation in alcohol.

Disadvantages of those loci concern a lengthy and costly development phase (especially when they must be identified and characterized in new taxonomic groups) (Yue *et al*., 2009) and different technical and/or molecular problems, which can involve genotyping errors.

There are several causes for genotyping errors. First, the appearance of shadows or stutter bands, due to slipping by Taq polymerase, which can complicate the interpretation of electrophoretic output. Second, the presence of null alleles, which is alleles that fail the amplification of a visible product, typically due to mutation at a priming site. Third, homoplasy, which is alleles of the same size but different lineages. Finally, large allele drop-out, which is preferential amplification of the "smaller" allele in a heterozygous genotype and the consequent low or lost amplification of the "larger" one. (De Woody, 2006; Selkoe & Toonen, 2006; Muneer *et al.*, 2014).

Genus *Anisakis* was investigated by microsatellite loci first in 2017, from the Authors Mladineo *et al*. They developed a panel of 11 microsatellites to study *A. simplex* (s. s.) and *A. pegreffii* species in the Mediterranean Sea, and to identify putative F1 hybrids between the

two considered taxa. The identification of hybrid specimens was performed at only one genetic marker, which is ITS region of rDNA (D'Amelio *et al*., 2000). Five groups were established on the total dataset, considering the results obtained by nuclear genotypes and mitochondrial haplotypes (mtDNA *cox-2* gene). Thus, the Authors defined a group of pure *A. pegreffii*, one group of pure *A. simplex* (s. s.) (from Norwegian Sea), two groups of hybrids with recombinant nuclear genotype and different matrilineage, and one group that shows discordance between nuclear and mitochondrial results (Mladineo *et al*., 2017a).

Nevertheless, the study of Mladineo *et al*., 2017a cannot be considered reliable for some reasons. First, the Authors employed the region of ITS of rDNA as genetic tool to identify recombinant genotypes between *A. pegreffii* and *A. simplex* (s. s.), despite the shared polymorphism between the species, and the consequent unsuitability of this marker, having already been proven (Mattiucci *et al*., 2016). Second, in that study the results about microsatellite genotyping of specimens considered in the dataset, both parental and recombinant ones, were not clear and comprehensive.

Therefore, hybridization phenomena in genus *Anisakis* can be considered still ambiguous and poorly investigated.

1.2 AIMS AND OBJECTIVES

In this work of thesis, a panel of seven microsatellite loci was developed on *A. pegreffii* species, and then successfully cross-amplified in the other species belonging to the *A. simplex* (s. l.) complex, i.e. *A. simplex* (s. s.) and *A. berlandi*. Additionally, five microsatellite loci developed from Mladineo *et al*. (2017a) were added in the study to the three species, in the aim to: *(i)* analyze the genetic structure of the three sibling species of *A. simplex* (s. l.) complex, by using a multigenotyping approach in their populations from both allopatric and sympatric areas (Study 1, 2 and 3); *(ii)* find new diagnostic genetic/molecular markers among

the three taxa, especially to perform a multilocus genotyping approach (Study 1 and 2); finally, *(iii)* investigate possible hybridization phenomena between the species *A. pegreffii* and *A. berlandi* in a sympatric area. (Study 3).

2. STUDY 1

NOVEL POLYMORPHIC MICROSATELLITE LOCI IN *ANISAKIS PEGREFFII* AND *A. SIMPLEX* (S. S.) (NEMATODA: ANISAKIDAE): IMPLICATIONS FOR SPECIES RECOGNITION AND POPULATION GENETIC ANALYSIS

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Abstract

The species of *Anisakis* constitute one of the most widespread groups of ascaridoid nematodes in the marine ecosystem. Three closely related taxa are recognised in the A. simplex (s. l.) complex, i.e. *A. pegreffii*, *A. simplex* (s. s.) and *A. berlandi*. They are distributed in populations of their intermediate/paratenic (fish and squids) and definitive (cetaceans) hosts. A panel of seven microsatellite loci (*Anisl 05784*, *Anisl 08059*, *Anisl 00875*, *Anisl 07132*, *Anisl 00314*, *Anisl 10535* and *Anisl 00185*), were developed and validated on a total of N = 943 specimens of *A. pegreffii* and *A. simplex* (s. s.), collected in fish and cetacean hosts from allopatric areas within the range of distribution of these parasite species. In addition, the locus *Anisl 7*, previously detected in those *Anisakis* spp., was investigated. The parasites were first identified by sequence analysis of the EF1 α-1 nDNA. The panel of the microsatellites loci here developed have allowed to: *(i)* detect diagnostic microsatellite loci between the two species; *(ii)* identify specimens of the two species *A. pegreffii*, *A. simplex* (s. s.) in a multi-marker nuclear genotyping approach; *(iii)* discover two sex-linked loci in both *Anisakis*

species and *(iv)* estimate levels of genetic differentiation at both the inter- and intra-specific level.

Key words: *Anisakis pegreffii*; *Anisakis simplex* (s. s.); EF1 α-1 nDNA; microsatellites; nuclear markers; population genetics; sex-linked loci.

2.1 INTRODUCTION

The species belonging to the genus *Anisakis* constitute one of the most widespread groups of ascaridoid nematodes in the marine ecosystem. These parasites involve various host species at different levels across the food webs: crustaceans as first intermediate host, fish, and squids acting as intermediate and/or paratenic ones and, finally, cetaceans as their main definitive hosts. Thus, their life-cycle completion is depending on the stability of marine trophic webs (Mattiucci and Nascetti, 2008; Mattiucci *et al*., 2018a).

To date, nine species belonging to the genus *Anisakis* have been genetically detected and morphologically described, worldwide. All those species possess distinct gene pools and are reproductively isolated; their existence as distinct phylogenetic units was demonstrated by various phylogenetic analyses (Valentini *et al*., 2006; Cavallero *et al.,* 2011; Mattiucci *et al.,* 2014). They have been also characterized on the basis of their different ecological features, having differential geographical distribution and host ranges (for a review see Mattiucci *et al.,* 2018a). Furthermore, two more gene pools, whose nomenclatural designation and formal description still need to be defined, have been detected genetically in the genus *Anisakis* – provisionally indicated as *Anisakis* sp. 1 and *Anisakis* sp. 2 – (Mattiucci *et al*., 2018a).

Three closely related taxa are so far included in the *A. simplex* (s. l.) complex: i.e. *A. pegreffii, A. simplex* (s. s.) and *A. berlandi* (Mattiucci *et al*., 2014). They are often distributed in metapopulations of their intermediate/paratenic (mainly pelagic/demersal fish and squids) and definitive (mainly Delphinoidea cetaceans) hosts from different geographical areas within the range of distribution of these parasites species. They have been found in co-infection in intermediate/transport hosts, such as several fish species and squids as well as in cetaceans from sympatric areas of distribution of the three sibling species (for a review, Mattiucci *et al*., 2018a). The third stage larvae of these *Anisakis* sibling species are also infecting a number of

fish species of high commercial-economic importance (Levsen *et al*., 2018). Humans may be accidental host, acquiring infection through consumptions of raw, marinated or undercooked fish and squids, infected by alive *Anisakis* spp. third stage larvae. Up to now two species, i.e. *A. pegreffii* and *A. simplex* (s. s.), are recognised as zoonotic parasites to humans (Umehara *et al*., 2007; Mattiucci *et al.*, 2017, 2018a; Guardone *et al*., 2018). Worldwide, there is an increasing recognition that the fish-borne zoonosis they provoke, known as anisakiasis, is an important emerging human disease (Chai *et al.*, 2005; Buchmann and Mehrdana, 2016; Guardone *et al.*, 2018; Mattiucci *et al*., 2018a). Risk factors for the transmission of the zoonotic species include consumption of locally harvested wild fish and traditional seafood home preparations. There is also an increasing interest in prioritizing fishborne zoonoses, not only in terms of their impact on human health but also in predicting the effects of climate and marine ecosystem changes. Thus, changes related to the ecology and epidemiology of actual *Anisakis* species may indirectly point at the consequences such changes may inflict on their natural hosts (both definitive and intermediate/paratenic ones) and the environment they live in (Mattiucci and Nascetti, 2008; Zarlenga *et al.*, 2014; Mattiucci *et al.*, 2018a).

Therefore, accurate identification of *Anisakis* species is essential for understanding their distribution, epidemiology, ecology, as well as their zoonotic potential in humans. It has been recognised that a multilocus genotyping approach is of great importance for species identification within the *A. simplex* (s. l.) complex (Mattiucci *et al*., 2016, 2018a). Applying the most appropriate sets of genetic/molecular markers in *Anisakis* species permits to: (1) disentangle cryptic species; (2) identify them at any stage of the life cycle; (3) establish phylogenetic relationships between related taxa; (4) provide an intraspecific population analysis; (5) estimate their genetic diversity and (6) assess possible hybridization and introgression phenomena between related species. Several genetic/molecular markers have so far been tested and validated in species recognition and to study the genetic structure of

Anisakis spp. (Nascetti *et al.,* 1986; D'Amelio *et al.,* 2000; Valentini *et al.,* 2006; Mattiucci *et al.,* 2014, 2016; Mladineo *et al*., 2017). Few attempts have been made to infer information on the genetic variation at the nuclear level of *A. pegreffii, A. simplex* (s. s.) and *A. berlandi* over a large range of populations and geographical areas. Indeed, investigations of the genetic structure of the species at population level was mostly based on allozymes (Mattiucci *et al*., 1997; Mattiucci and Nascetti, 2007), and, more recently, partially studied by means of mitochondrial markers (Baldwin *et al*., 2011; Blažeković *et al*., 2015; Mattiucci *et al.,* 2018b). The paucity of studies in *Anisakis* spp. at the population level was probably due to the perceived difficulty in obtaining informative nuclear markers for the recognition of these parasites at both species and population level.

A first attempt to isolate a panel of microsatellites, also known as simple sequence repeats (SSRs), for the species *A. simplex* (s. s.) and *A. pegreffii* was performed by Mladineo *et al.* (2017). The aim of the present study was to develop a novel panel of microsatellite loci, and validate them over a large number of specimens of *A. pegreffii* and *A. simplex* (s. s.) collected from several allopatric metapopulations within their geographical distribution range in order to: *(i)* provide further nuclear markers that allow to distinguish the two species in a multi-marker nuclear genotyping approach; *(ii)* evaluate the genetic variation of these parasite species inferred from SSRs analysis; *(iii)* estimate the genetic differentiation between the two species of the *A. simplex* (s. l.) complex, i.e. *A. pegreffii* and *A. simplex* (s. s.), as inferred from the SSRs and *(iv)* infer data on their population genetic structure.

2.2 MATERIALS AND METHODS

Collection of samples

A total of N = 943 specimens belonging to the two species *A. pegreffii* and *A. simplex* (s. s) were examined at DNA microsatellite loci. The specimens were collected as L3 larval stage

from intermediate/paratenic fish hosts and as L4-stage larvae and adults from cetacean species in sampling localities of the North East Atlantic Ocean, Mediterranean Sea, South West Atlantic Ocean and South West Pacific Ocean (Fig. 1 and Table 1). These localities are included in the geographical range of two species *A. pegreffii* and *A. simplex* (s. s.), from where they are reported in allopatry, according to Mattiucci *et al*. (2018a). Details concerning the sampling localities of the intermediate/paratenic (fish) and definitive hosts (cetaceans) of the two *Anisakis* species examined in this study are given in Table 1.

Some of the nematodes were obtained from the frozen collection of anisakids stored in the Section of Parasitology, Department of Public Health and Infectious Diseases of 'Sapienza University in Rome'; whereas other L3 samples were collected during the years 2013–2016 from fish in the framework of the Project PARASITE and belong to the PARASITE-Biobank (Gonzáles *et al.*, 2018). Node at the Section of Parasitology (Sapienza-University). The collection of *Anisakis* spp. adults was undertaken during the years 2011–2012 from stranded cetaceans, i.e. the pilot whale, *Globicephala melas* Traill, the Risso's dolphin *Grampus griseus* (Cuvier) and the Hector's dolphin *Cephalorhynchus hectori* (van Beneden) in the Southern Pacific Ocean (off the coast of New Zealand), the striped dolphin, *Stenella coeruleoalba* (Meyen), stranded off the Italian coast, and the harbour porpoise, *Phocoena phocoena* (Linnaeus) from the English Channel (Table 1).

Nematodes collected from the stomach of their hosts were washed in saline solution and then preserved in 70% alcohol. The nematodes collected from cetacean definitive hosts were first distinguished as L4-stage larvae and adults, then adults between females and males, according to the main morphological features diagnostic between sexes (Mattiucci *et al.*, 2018a), by the use of an optical microscope at ×100–400 total magnification. The central part

of the worm's body was thus used for the molecular analysis, while the cephalic and caudal ends were stored for male and female discrimination.

DNA extraction

Total DNA was extracted from each *Anisakis* larva L3 in toto, and from a tissue portion of 2mg from each adult and L4 larval specimen (Table 1). The cetyltrimethylammonium bromide method (CTAB) was used as the extraction method (details in Mattiucci *et al*., 2014). DNA obtained was quantified by using the Qubit™ dsDNA HS Assay Kit with Qubit 2.0 (Invitrogen™) (Sambrook and Russell, 2001). First, aliquots with a standard concentration of 10 ng ml−1 were prepared from a pooled DNA extraction of N = 10 specimens of *A. pegreffii* for developing the genome library, and then on 15 unrelated specimens of the same species for testing the new markers. Later on, DNA from each specimen considered in this study was used to perform the SSRs analysis.

Genetic identification of* Anisakis *spp. specimens

All the specimens used (N = 943) to validate the novel DNA microsatellite loci here developed were previously identified at the species level by molecular identification, using sequences analysis at the EF1 α-1 nDNA (Mattiucci *et al.,* 2016).

The amplification of EF1 α-1 nDNA was performed using the primers EF-F (5′-TCC TCA AGC GTT GTT ATC TGT T-3′) and EF-R (5′-AGT TTT GCC ACT AGC GGT TCC-3′) (Mattiucci *et al*., 2016) under the following conditions: 94 °C for 3 min (initial denaturation), followed by 35 cycles at 95 °C for 45 s (denaturation), 58 °C for 40 s (annealing) and 72 °C for 1 min, with a final post-amplification step at 72 °C for 10 min.

PCR amplification was carried out in a volume of 25 μL containing 0.5 μL of each primer 10 mM, 2.5 μL of MgCl2 25 mM (Promega), 1.5 μL of PCR buffer 5× (Promega),

0.08 mM of DMSO, 0.5 μL of dNTPs 10 mm (Promega), 5 U of HotStart Go-Taq Polymerase (Promega) and 2 μL of total DNA (Mattiucci *et al*., 2016), for the EF1 α-1 nuclear gene.

Microsatellite (SSRs markers) development and primers selection

For the development of SSRs markers, using the whole-genome sequencing via Illumina (MiSeq platform), a sub-samples of pooled 10 specimens of the species *A. pegreffii* was first selected for the genomic library development. In order to avoid parasite tissue contamination with the fish host DNA, alive *A. pegreffii* larvae collected from the fish were first washed using physiological solution, plus acetic acid at 4%. Then, the larvae were maintained in vitro culture in a Petri dish, with physiological solution for 24 h, at 6–7 °C; the larvae were thus transferred in single 1.5 mL tubes, containing PBS 1× solution, for 72 h (the PBS 1× solution was changed daily); finally, *Anisakis* larvae were washed several times with ultrapure water, and stored for the following DNA extraction. 'Pure' DNA from those *A. pegreffii* treated larvae was extracted and first tested by using the barcode primers for several fish species, to be sure that DNA from the parasite was not contaminated with that from the host.

Size-selected fragments from genomic DNA of the considered species (*A. pegreffii*) were enriched for SSRs content by using magnetic streptavidin beads and biotin-labelled GTAT, GATA, repeat oligonucleotides. The SSRs-enriched library was analysed on an Illumina MiSeq platform using the Nano 2 × 250 v2 format. After assembly, 19.332 contigs/singlets were screened and 785 contigs/singlets contained a microsatellite insert with a tetra- or a trinucleotide of at least six repeat units, were considered; finally, suitable primer design was possible in 429 microsatellite candidates. The SSR-enriched library obtained from those pooled samples of *A. pegreffii* was also checked with the genome of *A. simplex* (s. l.) (PRJEB496) hosted at WormBase Parasite (Howe et al., 2016), assembled by the Parasite

Genomic Group at the Welcome Trust Sanger Institute, UK (http://parasite.worm-base.org/Anisakis_simplex_prjeb496/Info/Index).

A total of seven *A. pegreffii* microsatellite loci were selected and designated as: *Anisl 05784, Anisl 08059, Anisl 00875, Anisl 07132, Anisl 00314, Anisl 10535, Anisl 00185* (Table 2). Primers were designed from the flanking sequences to produce a 69–250 bp amplicons and to have an optimal annealing temperature near 56 °C (Table 2). Alleles at each locus are referred to by their size in base pairs, as determined by GeneMapper software (Table 2). The primers for the seven best loci were chosen and scored, at first, on 15 specimens of *A. pegreffii* collected from different fish hosts and geographical areas, for testing them in a larger set of individuals and for consistency in PCR amplification. All markers revealed a number of alleles ranging between 5 and 12 in those first analysed specimens of *A. pegreffii* used for SSRs development. The allele size was determined by the use of an ABI3730 instrument, using GeneScanTM-500 LIZ Size Standard.

The above-selected novel SSRs loci (Table 2) were then used to evaluate their success in cross-species amplification of *A. simplex* (s. s.) species. The markers developed for the specimens of *A. pegreffii* successfully amplified DNA obtained from *A. simplex* (s. s.) samples. Therefore, all the larval and adult specimens corresponding to the two species *A. pegreffii* and *A. simplex* (s. s.) reported in Table 1 were analysed with the novel panel of SSRs markers.

PCR amplification of SSRs markers, and genotyping

Two multiplex PCR amplifications (with four and three couple of primers respectively) were optimized to be 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 labelled forward and reverse primers each (Table 2). The following cycling protocols

were used for the amplification both for the two 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. The features for the seven loci selected, i.e. the repeat motif, the bp size, the primer sequences and its labelling dye, are reported in Table 2.

Amplified PCR products were genotyped by an external Company (Macrogen service). An additional microsatellite marker, i.e. the *Anisl 7* from Mladineo *et al*. (2017), was also tested on all those specimens of the two *Anisakis* spp. considered in the present study (Table 1); its analysis was included in the Multiplex no. 2 and the forward primer was labelled with the NED dye (Table 1); specific PCR conditions used for this locus were those previously described in the text for multiplex 2 (see above).

Finally, the alleles obtained from the electropherograms were identified and binned using the software Genemapper v.4.1 (Applied Biosystems, USA). Genotyping errors generally associated with microsatellite analysis, such as stutter bands, the presence of null alleles and large drop-out alleles, were verified by using the software MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004). Patterns of tri- and tetrallelic peaks in the female individuals, as possible results of tissue contamination from sperm males fecundation, were not found.

Genetic data analysis

All samples tested in this study at the panel of microsatellite markers were previously identified by sequences analysis obtained at the EF1 α-1 nDNA partial gene (Mattiucci *et al*., 2016). The sequences obtained in the EF1 α-1 nDNA region were aligned by using Clustal X (version 2.0) software (Larkin *et al.*, 2007) with those from the same species previously

obtained (Mattiucci *et al.*, 2016), in order to detect those fixed diagnostic nucleotide positions allowing to discriminate *A. simplex* (s. s.) and *A. pegreffii* (Mattiucci *et al*., 2016).

The observed heterozygosity (H_o), the expected heterozygosity (H_e), the mean number of alleles per locus (A) were calculated by ARLEQUIN version 3.5 (Excoffier and Lischer, 2010). The occurrence of the expected Hardy–Weinberg equilibrium (HWE) at each locus was assessed by means of exact tests, as implemented in the software ARLEQUIN version 3.5 (Excoffier and Lischer, 2010). Significance levels were adjusted using the sequential Bonferroni correction for multiple tests (Rice, 1989). The fixation index (F_{IS}) from the genetic data sets obtained at the SSRs loci in the analysed populations of the two *Anisakis* species, was estimated using ARLEQUIN version 3.5 (Excoffier and Lischer, 2010), and F_{ST} (Weir and Cockerham, 1984) by FSTAT version 1.2 (Goudet, 1995). We used analysis of molecular variance (AMOVA) as implemented in ARLEQUIN version 3.5, with 1000 permutations, on the genetic data sets obtained in the populations of *A. simplex* (s. s.) and *A. pegreffii*. Nei's distance matrix (Nei, 1978), was calculated from the allele frequencies estimates, by using BIOSYS 2.0 software program (Swofford and Selander, 1997). An UPGMA (Unweighted Pair Group Method with Arithmetic Mean) was generated by using PHYLIP software (Felsenstein, 1993), based on Nei's distance values. In addition, a multivariate-based method for inference of population structuring was based on non-metric multidimensional scaling (nMDS) ordination plot, by using allele frequencies observed at the microsatellite loci in all the tested populations of the two parasites species, based on the calculation of Euclidean distance. The nMDS-plot for the data set was performed by the package Vegan Community Ecology Package, R version 2.5-4 (Oksanen, *et al*., 2019).

Finally, a Bayesian clustering algorithm elaborated by the program STRUCTURE 2·3·3 (Pritchard *et al.*, 2000) was used to assign each analysed specimen to the species *A.*

pegreffii or *A. simplex* (s. s.), as based on its genotype showed at those SSRs markers, plus the EF1 α-1 nDNA region. The same approach allowed to identify any eventual instances of gene exchange between the two species. STRUCTURE is a model-based procedure that uses individual multi-locus genotypes to identify the optimal number of clusters (K) in a dataset, by minimizing the resulting Hardy-Weinberg and linkage disequilibria. The analysis was run setting the predefined number of clusters between 1 and 9 (i.e. the number of sampling areas in our dataset). 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 optimality criteria (Evanno *et al.*, 2005).

2.3 RESULTS

Identification of Anisakis *spp. specimens by sequencing of the EF1 α-1 nDNA region*

A total of N = 943 larval and adult specimens of *Anisakis* spp. (Table 1) to be used for the microsatellites development and validation, were first identified to species level by sequences analysis of the nuclear EF1 α-1 nDNA partial region. A fragment of 409 bp of the EF1 α-1 nDNA region was obtained for all 943 specimens analysed. It revealed the presence of two diagnostic nucleotide sites in positions were 186 and 286 (as indicated in Mattiucci *et al.* (2016)), showing T and C in *A. pegreffii* but C and T in the parental taxon *A. simplex* (s. s.), respectively. According to this analysis, N = 451 specimens were assigned to species *A. pegreffii*, whereas N = 492 were assigned to *A. simplex* (s. s.) (Table 1). Patterns of heterozygote genotypes, i.e. showing two overlapping C/T peaks, were not found among all the presently analysed individuals of the two species. Sequences of the EF1 α-1 nDNA region

were deposited in GenBank under the accession numbers from MK032267 to MK032270 for *A. pegreffii*, and from MK032271 to MK032275, for *A. simplex* (s. s.).

Genetic diversity of SSRs in* A. pegreffii *and* A. simplex *(s. s.)

The number of individual specimens genotyped at the eight scored SSRs varied from a minimum of N = 77 to a maximum N = 164 worms of *A. pegreffii* and from N = 57 to N = 176 from the selected populations of *A. simplex* (s. s.) (Table 3). For some markers, such as the locus *Anisl 7*, there was a small number of worms in each population of the two analysed parasite species that repeatedly failed in the genotyping. Thus, the total number of nematodes of the two species tested at the locus *Anisl 7*, was N = 713, with respect to the total sample (N = 943) (Tables 1 and 3). However, a high representative number of specimens from each population of the two species from the selected geographic areas was available at that locus to be used in the subsequent genetic data analyses (Table 3).

There were some differences in the overall genetic diversity values of the different populations of the two species, based on both the mean number of alleles per locus, and values of expected heterozygosity per locus (H_e) (Table 3). All the microsatellite loci scored in the present study were polymorphic, with the total number of alleles varying in *A. pegreffii* between 2 (such as those observed at the locus *Anisl 7* in the population from the Adriatic Sea (AD)) and 13 (such as those found in the population from New Zealand (NZ) at the locus *Anisl 05784*) (Table 3). In *A. pegreffii*, the mean value of alleles per locus (A) was A = 8.21. In *A. simplex* (s. s.), the total number of alleles was found to vary between 3 (such as those found at the locus *Anisl 10535* in the population from Baltic Sea, BA) and 13 (such as those observed at the loci *Anisl 05784* and *Anisl 00314* in the population from English Channel, EC) (Table 3). In *A. simplex* (s. s.), the mean value of alleles per locus (A) was A = 8.27.

Deviations from the Hardy-Weinberg Equilibrium (HWE) at each locus were tested in each selected population of the two species (Table 3). Generally, positive values of F_{IS} indicated an excess of homozygote genotypes at the selected loci; whereas, negative values indicate an excess of heterozygotes from the expected HWE (Fig. 2A). No significant deviations between observed (H_o) and expected (H_e) heterozygosity resulted at loci *Anisl 10535, Anisl 07132, Anisl 05784, Anisl 08059* and *Anisl 00875*, in all the analysed populations of *A. pegreffii* and *A. simplex* (s. s), with not-significant departure from the HWE (Table 3). Some significant HWE deviations occurred in *A. simplex* (s. s.) at the locus *Anisl 00185*; conversely, the same locus did not show significant deviation between H_o and H_e in the examined populations of *A. pegreffii* (Table 3) (Fig. 2A). However, due to the high significant departure from HWE observed at that locus in populations of the species *A. simplex* (s. s.), this marker was not taken into account when population genetic data have been considered.

Sex-linked microsatellite loci

Statistically high significant departures from the HWE in all the analysed populations of *A. pegreffii* and *A. simplex* (s. s.) were observed also at loci *Anisl 00314* and *Anisl 7*. The two loci showed a marked excess of homozygotes in all populations (Table 3), with positive F_{IS} value (Fig. 2A). Interestingly, when splitting the genotypes at the loci *Anisl 00314* and *Anisl 7* found in both *A. pegreffii* and *A. simplex* (s. s.) into adult male and female worms, it resulted that the male worms of the two species were homozygous at those loci (F_{IS} = 1) (Fig. 2B). Therefore, the loci *Anisl 00314* and *Anisl 7* appear to be sex-linked loci in both species, likely because of the hemizygosity state (i.e. diploid individuals in which there is only one allele present at the sex-linked loci) of the males in both the *Anisakis* spp., as X-linked inheritance. Indeed, no significantly departures from the HWE were found between the expected

homozygous and heterozygous females at the *Anisl 7* in the two *Anisakis* spp. (Table 4). Analogously, no significant departure from the HWE was found at the locus *Anisl 00314* in all the populations of *A. pegreffii* (Table 4). While, at the same locus, the populations of *A. simplex* (s. s.) showed a significant excess of homozygous genotypes, also after accounting only the genotypes from female worms (Table 4). The reason for this partial departure is not known, but a possibility could be that some alleles failed to amplify in the species *A. simplex* (s. s.), thereby increasing the apparent homozygosity of the female populations, or probably the phenomenon was due to null alleles in *A. simplex* (s. s.) at this locus. As a consequence of the HWE departures found in *A. simplex* (s. s.) (Table 4), the locus *Anisl 00314* was excluded from the subsequent population genetic analysis.

Whereas, to not exclude genetic data set obtained at the other sex-linked locus (i.e. *Anisl 7*) from the subsequent population genetic analysis, the most reliable estimates of allele frequencies of the two parasite species were calculated only in adult specimens, according to the sex-linked genetic model estimate, assuming: (i) the hemizygosity of the males in the two *Anisakis* species; (ii) their adult female counterparts, as biallelic nematodes at the sex-linked loci. Thus, only adult nematodes of *A. pegreffii* collected in *S. coeruleoalba* from Adriatic Sea (AD), in *G. melas* and *C. hectori* from New Zealand waters, as well as adult worms of *A. simplex* (s. s.) recovered in *Phocoena phocaena* from English Channel (EC), were accounted for the allele frequencies estimates (Table 5). Indeed, because the gender assignment was not possible for larval individuals, as a consequence, the populations of the two *Anisakis* species including only larvae (see Table 1) were not included in the allele frequencies estimate.

Identification of* Anisakis *spp. specimens by using SSRs loci

Interestingly, among the SSRs loci studied, *Anisl 10535*, *Anisl 05784*, *Anisl 00875*, *Anisl 08059* And *Anisl 7* have shown a certain differential frequency distribution at some alleles in

the two species (Table 5). In particular, the locus *Anisl 7* had up to eight different alleles in populations of *A. simplex* (s. s.); it resulted to be less polymorphic in *A. pegreffii*, showing up to four alleles in the population from New Zealand (Table 5). However, at this locus all the alleles found in *A. simplex* (s. s.) populations were clearly distinct from those observed in *A. pegreffii*. In other words, *Anisl 7* showed alternative alleles in *A. pegreffii* and *A. simplex* (s. s.), thus suggesting that this is a diagnostic locus (100%) between the two parasite species (Table 5).

Individual *Anisakis* worms were assigned to either *A. pegreffii* or *A. simplex* (s. s.) based on a Bayesian clustering algorithm which is implemented in STRUCTURE 2.3.3 (Pritchard *et al*., 2000), according to their genotypes observed at the five SSRs loci, *i.e. Anisl 10535, Anisl 05784, Anisl 00875, Anisl 08059* and *Anisl 07132* (Fig. 3A). Because of the hemizygosity of the males at the sex-linked locus *Anisl 7*, and the presence of null alleles found at the locus *Anisl 00185* in *A. simplex* (s. s.), genotypes found at those loci were obviously excluded from the STRUCTURE analysis. Using both the highest ln probability and the delta-K (Evanno *et al*., 2005) optimality criteria, the STRUCTURE analysis indicated K = 2 as the clustering option which best fitted the data set (Suppl. Fig. 1A and B).

Thus, considering those five microsatellite loci with that clustering option, all individuals from samples TY (Tyrrhenian Sea), AD (Western Adriatic Sea), NZ (New Zealand coast) and AR (Argentine coast) were assigned with high percentage of assignment to *A. pegreffii* (Fig. 3A). Similarly, all individuals from sampling locality NS (North Sea), Norwegian Sea (NW), English Channel (EC), Baltic Sea (BA) and Grand Sole Bank (GS) were referred to *A. simplex* (s. s.), as inferred from the same Bayesian assignment/cluster analysis (Fig. 3A). Finally, when taking into account the genotypes of all the examined individuals found at those five nuclear loci, plus those observed at the diagnostic positions of

the EF1 α-1 nDNA partial region, as clustering option by STRUCTURE, all the individuals were assigned to the distinct species (*A. pegreffii* or *A. simplex* (s. s.)), with high membership level (>99%) (Fig. 3B).

However, higher percentage of assignment (>99%) was even obtained when based on the genotypes observed only in the female specimens tested at six SSRs loci (including also the females genotyped at the X-linked locus *Anisl 7*, but excluding the locus *Anisl 00185* due to null alleles) (Suppl. Fig. 2).

No genotypes/individuals of mixed ancestry between the two species (such as F1 hybrids), i.e. with expected Q-value = 0.50, were found in this analysis, thus suggesting that all individuals *Anisakis* presently analysed from allopatric populations of the two species, were "pure parental" specimens of *A. pegreffii* or *A. simplex* (s. s.). On the other hand, no heterozygotes at the diagnostic positions of EF1 α-1 nDNA, nor at the diagnostic alleles observed at the *Anisl 7* locus, were found in the samples.

Genetic differentiation at the inter and intra-specific level, as inferred from SSRs loci

Excluding the SSR locus (i.e. *Anisl 00185*) affected by null alleles in *A. simplex* (s. s.), and the sex-linked loci *Anisl 7* and *Anisl 00314* due to hemizygosity of males in both species of *Anisakis* and the presence of null alleles in the latter SSR locus, the remaining loci showed adequate genetic diversity for population-level genetic analysis. The AMOVA analysis of the five nuclear markers showed that in both the species most of the variance was significantly allocated within individuals (≈87% and ≈89%), with F_{IT} = 0.12 and F_{IT} = 0.10, respectively, in *A. pegreffii* and *A. simplex* (s. s.). Further, a notable variation was found among individuals within populations. AMOVA provided also moderate significant variation among infrapopulations of *A. pegreffii;* less significant was the percentage of variation among the populations of the species *A. simplex* (s. s.) (Suppl. Table 1a, b).

Pairwise F_{ST} values were calculated for all the population pairs of the two *Anisakis* species, to quantify the extent of genetic substructuring between different populations (Table 6). Based on those five SSRs loci considered, a significantly high level of genetic differentiation resulted at the interspecific level, between the populations of *A. pegreffii* and the populations of *A. simplex* (s. s.) (on average $F_{ST} \approx 0.29$ $P \ll 0.001$) (Table 6), with the Adriatic Sea (AD) population of *A. pegreffii* diverging the most from the present *A. simplex* (s. s.) populations.

At the intraspecific level, i.e. when analysing the microsatellite data separately for each species including all stages, much lower values of genetic differentiation was observed. No significant genetic differentiation was observed between conspecific populations of larvae *vs* adults from fish and cetacean hosts being, $F_{ST} \approx 0.008$ and $F_{ST} \approx 0.004$ respectively, in *A. pegreffii* and *A. simplex* (s. s.). At the interpopulation level, the genetic differentiation among populations of *A. simplex* (s. s.) from NE Atlantic waters was, on average, $F_{ST} \approx 0.008$. The highest F_{ST} value = 0.021 ($P < 0.001$) was found between the Baltic Sea (BA) population of *A. simplex* (s. s.) vs the North Sea (NS) population of the same species (Table 6).

In *A. pegreffii*, low level of differentiation was found between pairs of populations geographically close to each other, such as the two samples from the Mediterranean Sea (i.e. TY *vs.* AD, $F_{ST} = 0.002$), or between the austral populations of *A. pegreffii* from off the New Zealand and Argentine coast ($F_{ST} = 0.007$) (Table 6). Whereas, higher significant genetic sub-structuring (on average, $F_{ST} \approx 0.060$ $P < 0.001$) seems to exist when comparing the Austral populations (NZ and AR) of *A. pegreffii* with the Mediterranean ones (TY and AD) (Table 6).

The genetic distance (D_{Nei}) values were based on the allele frequencies observed at five SSRs loci (*Anisl 10535, Anisl 05784, Anisl 00875, Anisl 08059* and *Anisl 07132*). Indeed, in the estimation of the genetic distance values at population level, it was not possible to

include the sex-linked locus *Anisl 7* due to the fact that allele frequencies of adult specimens were available only in three populations of the two *Anisakis* species (i.e. NZ, AD, and EC) (see Tables 1 and 5).

At the interspecific level, the value of D_{Nei} observed among the currently analysed populations of the two species was on average $D_{Nei} \approx 0.91$, as based on five SSRs loci. Genetic distance values between the two *Anisakis* spp. ranged from $D_{Nei} = 0.670$ (BA *vs* NZ) to $D_{Nei} = 1.094$, observed between the population of *A. simplex* (s. s.) from Norwegian Sea (NW) *vs A. pegreffii* from Adriatic Sea (AD) (Table 6). At the intraspecific level, values of D_{Nei} were clearly lower. In *A. pegreffii*, on average, the value resulted $D_{Nei} \approx 0.15$ between the Austral and Boreal populations of the species (Table 6). Conversely, low level of genetic differentiation was found among the Mediterranean populations of *A. pegreffii* ($D_{Nei} = 0.005$). Analogously, a high genetic similarity was observed among the NE Atlantic populations of the species *A. simplex* (s. s.) (on average, $D_{Nei} = 0.021$), with the population from the English Channel being the most distant population (on averages $D_{Nei} = 0.037$) from the other conspecific ones of the NE Atlantic Ocean (Table 6).

The genetic relationships among the populations and species of the two *Anisakis* taxa are illustrated in the UPGMA cluster (Fig. 4), as inferred from Nei's genetic distance values (Table 6), and in the nMDS analysis (Fig. 5) obtained from the allele frequencies calculated at five microsatellite loci (Table 5). Both analyses agreed in depicting a high level of genetic divergence between the analysed populations of *A. pegreffii* and *A. simplex* (s. s.). On the other hand, they were congruent in showing that the populations of *A. pegreffii* and *A. simplex* (s. s.) are clustering in two well distinct clades, corresponding to the two biological species. Additionally, they were also concordant in showing close genetic relationship and similarity between the two Austral populations of *A. pegreffii* (NZ and AR) which are clustering in the

same sub-clade (UPGMA analysis) and represent close clusters in the nMDS analysis, as well. However, marked substructuring of the Austral populations (NZ and AR) of *A. pegreffii* compared to populations of the same species from Boreal areas (i.e. from Tyrrhenian Sea, TY and Western Adriatic Sea, AD) was shown in the multilocus plot analysis (Figs 4 and 5).

2.4 DISCUSSION

Genetic diversity of microsatellites in A. pegreffii *and* A. simplex *(s. s.)*

Although microsatellites have been extensively used to determine population structure of marine species, so far they have received little attention in marine parasites. In recent years, microsatellites analysis of trematode parasites has been used to identify origin of the steelhead trout *Oncorhynchus mykiss* (Criscione *et al.*, 2006), or to show congruent population genetic pattern of parasite species (i.e. *Plagioporus shawi*) and its salmonid hosts (*Oncorhynchus* spp.) (Criscione and Blouin, 2007). Despite their potential utility, microsatellites as genetic markers for parasitic nematodes have been not much explored (Johnson *et al.*, 2006; Criscione *et al.*, 2007; Redman *et al.*, 2008; Temperley *et al.*, 2009; Betson *et al.*, 2011; Reid *et al*., 2012; Patrelle *et al.*, 2014; Rabelo *et al.*, 2017; Greeff *et al.*, 2018). This may be explained by some complexity when using microsatellites, which could arise from potential homoplasy, i.e. the independent mutation of microsatellite markers of the same size, and the occurrence of null alleles (Grillo *et al*., 2006; Criscione *et al.*, 2007; Redman *et al*., 2008; Glenn *et al.*, 2013).

In the present study, seven novel nuclear polymorphic loci have been developed and characterized as nuclear markers by next-generation sequencing approach in the marine parasite species *A. pegreffii*. The primer pairs have allowed the robust amplification from each single individual worm, and a clear unambiguous genotype detection. Further, they have cross-species amplified the same SSRs in the sister taxon *A. simplex* (s. s), as well as in *A.*

berlandi (Mattiucci et al., pers.com.). However, among the novel microsatellite loci here scored, the loci *Anisl 00185* and *Anisl 00314* were found to be affected by null alleles in the species *A. simplex* (s. s.). The high frequency of null alleles generally observed in SSRs markers is likely to reflect polymorphism of flanking sequence in form of SNPs and/or indels, such as those frequently found in other nematode species (Temperley *et al*., 2009). Also, when a mutation occurs in the primer region, some individuals could fail in one allele amplified (Selkoe and Toonen, 2006). This would have been occurred just in the species *A. simplex* (s. s.) because of the fact that the SSRs primers have been here first selected in the species *A. pegreffii*. In many other taxonomic groups, PCR amplifications are known to fail with higher likelihood when heterospecific SSRs primers are employed (Lowe *et al*., 2002). However, the high significant HWE deviations found at the locus *Anisl 00185* and *Anisl 00314* in the species *A. simplex* (s. s.) (Table 3 and 4) was taken into account, excluding those loci when population genetic data sets of that species were being analysed.

The novel markers detected in the present study have been validated on a very large number of individuals from allopatric populations of the two species (i.e. N = 451 of *A. pegreffii* and N = 492 of *A. simplex* (s. s.)). Further, a large number among those nematodes of A. pegreffii (N = 372) and of *A. simplex* (s. s.) (N = 341) were characterized at *Anisl 7* SSR marker. The last one was firstly scored by Mladineo *et al.* (2017), in the aim of developing microsatellites loci in species of the *A. simplex* (s. l.) complex. However, the authors scored at that locus only very few specimens for *A. simplex* (s. s.), and from a single population of *A. pegreffii* from the Eastern Adriatic Sea. Thus, on the basis of results obtained on a too low number of specimens analysed by the authors, the locus *Anisl 7* was not pointed out as a valuable diagnostic marker between the two species.

X-associated SSRs loci in* Anisakis *spp.

An interesting result was that in the species *A. pegreffii* and *A. simplex* (s. s.) the two loci *Anisl 00314* and *Anisl 7* are located on sexual X chromosome(s), thus being sex-linked. Indeed, it was demonstrated that male specimens of *A. pegreffii* and *A. simplex* (s. s.) are hemizygous at both the SSRs loci for several distinct alleles. However, it was also found that the locus *Anisl 00314* was affected by null alleles in *A. simplex* (s. s.), because of the significant HWE departures observed, even when excluding male genotypes from the test (Table 4).

The finding of X-associated SSRs loci was not detected by Mladineo *et al.* (2017), who retained that the occurrence of excess of homozygote genotypes found at the same *Anisl 7* locus in the two *Anisakis* species was related to the fact that it was affected by null alleles.

The finding of two sex-linked nuclear SSRs loci in species of the genus *Anisakis* represents the first evidence in anisakid nematodes. The X-linked SSRs loci have been previously reported in other ascaridoid nematodes, such as in *Ascaris* spp. (Criscione *et al*., 2007), as well as several other nematodes (Johnson *et al.*,2006). It was indeed demonstrated that *A. suum* has 19 autosomes and 5X chromosomes (2n = 38A + 10X in females, 38A + 5X in males) (Müller and Tobler, 2000). The study on chromosomes of anisakid worms has never been carried out. However, the finding of the sex-linked loci could be due to the fact that male specimens belonging to *Anisakis* spp. likely possess XO sexual karyotype, as other ascarids (Müller and Tobler, 2000). According to Criscione *et al.* (2007), the number of sex-linked SSRs in the terrestrial ascarid *A. lumbricoides* may represent 20% of the parasite's total genome. Similarly, Johnson *et al*. (2006) found that 3 of 21 SSRs loci were sex-linked in Trichostrongylus tenuis. In the present study, it seems that the percentage of SSRs X-linked loci would be around 25% in species of the *A. simplex* (s. l.).

Paradoxically, in spite of the hemizygosity of male nematodes, those gender-associated SSRs markers discovered in the present study could help in revealing an alternative mode of sex determination in those *Anisakis* species, when other gender features are not otherwise evident. Indeed, the two X-linked loci (i.e. *Anisl 7* and *Anisl 00314*) can be considered as possible markers for the identification of an individual's female sex, at any life-history stage (even as L3 larva or immature worm), when it shows a heterozygote genotype at one or both the two loci. According to the two sex-linked loci so far discovered, sex determination would be inferred at least in the species *A. pegreffii*, not affected by null alleles at the locus *Anisl 00314* (Table 4). However, despite their utility in gender markers, only two loci are not enough in female recognition, taking into account the loss of homozygote genotypes in females at those X-linked loci. Further X-linked SSRs loci which will be discovered in future analysis in the members of the *A. simplex* (s. l.) would help to increase the probability of a correct gender assignment in these nematodes.

***Utility of microsatellite markers in the identification of* A. pegreffii *and* A. simplex (s. s.)**

Another aspect highlighted by this study was the finding that the present SSRs loci are of potential value in the discrimination of the two *Anisakis* species, when using a Bayesian clustering approach. Interestingly, in the present Bayesian analysis by STRUCTURE of genotypes observed at five loci, all the considered *Anisakis* specimens were assigned to *A. pegreffii* or *A. simplex* (s. s.) at very high assignment rate (>99%) (Fig. 3A). In addition, when including in the same analysis the genotypes inferred from another diagnostic nuclear marker, i.e. from the EF1 α-1 nDNA, percentage of assignment reached 100% in almost all individuals, while in others the percentage of correct assignment was, again, >99% (Fig. 3B).

Among the scored SSRs markers, it was found that some of the polymorphic loci seem to share the same alleles at several loci in *A. pegreffii* and *A. simplex* (s. s.), but often with

significant different frequency proportion (such as *Anisl 10535*); while other loci show alternative alleles in the two species, such as the locus *Anisl 7* (Table 5). Thus, the last one can be retained as a 100% diagnostic locus to separate the two species, as inferred on the present study, validated by results obtained on a large number of individuals (N = 713) of both species. A molecular/genetic key of potential value in the identification of the specimens belonging to the two species, based on the alleles scored at some SSRs loci (which can be combinable in a single one multiplex PCR analysis), has been also here proposed (Table 7).

In the present genetic analysis, no individuals showing evidence of mixed ancestry genotypes were detected between the two species collected from allopatric areas by the Bayesian multilocus approach. On the contrary, it has been demonstrated that misidentifications of specimens of parental *A. pegreffii* and *A. simplex* (s. s.), and their hybrid categories could result from the identification derived from only a single nuclear marker, i.e. the ITS region of the rDNA gene locus (Mattiucci *et al*., 2016). These findings raise the question in the reliability of the ITS nuclear marker in the species identification of these two sibling species, and underline the usefulness and validity of a multilocus genotyping approach. Combining the use of several nuclear markers, such as those presently outlined, would greatly increase the informative value of future analyses in the aim to clarify patterns of hybridization and introgression events in closely related taxa of the *A. simplex* (s. l.) complex (Mattiucci *et al*., 2018a).

***SSRs markers for population genetics analysis of* A. pegreffii and A. simplex (s. s.)**

The panel of microsatellite markers here identified showed to be suitable for population genetics analysis of *A. simplex* (s. s.) and *A. pegreffii*, as observed by examining the degree of genetic variation between populations of the two species collected from allopatric areas of the parasites.

Using different clustering approaches (STRUCTURE, UPGMA and nMDS analyses) as inferred from the analysis of microsatellite loci, it was demonstrated that the populations analysed in the present study, despite wide host range and geographically distant sampling localities, were strongly associated with two distinct panmictic units, that correspond to the two species *A. pegreffii* and *A. simplex* (s. s.).

At the intraspecific level, a moderate genetic differentiation (on average, F_{ST} = 0.060) was found among the Boreal populations of *A. pegreffii* from the Adriatic and Tyrrhenian populations of the Mediterranean Sea, and their conspecific ones from the Austral region off New Zealand and the Argentine coast. This finding is in accordance with similar values of genetic differentiation previously found at the allozyme level: indeed, in *A. pegreffii* a $G_{ST} \approx$ 0.045 value was recorded between the Adriatic Sea and New Zealand samples (Mattiucci *et al*., 1997). Also, a similar trend was observed at the mitochondrial gene (mtDNA cox-2 sequences data analysis) level (Blažeković *et al*., 2015). Indeed, the last authors have found that a significant sub-structuring was reported between mtDNA cox2 sequences data sets of the western and eastern populations of *A. pegreffii* of the Pacific Ocean, obtained from sequences deposited in Genbank, with respect to population of the same species collected by those authors from the Adriatic Sea. On the contrary, the same authors (Blažeković, *et al.,* 2015) did not report significant genetic sub-structuring between Adriatic Sea and other Mediterranean populations of *A. pegreffii*; this last finding seems to be congruent with the low level of genetic differentiation here estimated at the SSRs level, between the populations of the Adriatic and Tyrrhenian Sea waters. The finding of a genetic substructuring of *A. pegreffii* from Austral and Boreal regions suggests that geographic distance would restrict the gene flow between 'antipodean' populations of this parasite species. However, based on the F_{ST} values observed at the five SSRs loci, the species *A. pegreffii* showed a high-gene flow value (Nm = 7.8) between the analysed Boreal and Austral populations. This resulted to be at

similar degree as that previously found at allozymes level (Mattiucci *et al.*, 1997; Mattiucci and Nascetti, 2008). Different populations and species of cetaceans, among those analysed in the present study, would be responsible for shaping the observed genetic sub-structuring of Boreal and Austral subpopulations of *A. pegreffii*, as also suggested in our previous studies (Mattiucci *et al.*, 2014), and by other authors (Blažeković *et al.*, 2015). The low Nei's *D* and F_{ST} values observed between the studied populations of *A. simplex* (s. s.), revealed a low level of genetic sub-structuring among the analysed samples collected from different basin waters of the NE Atlantic Ocean. This finding was in accordance with previous observations obtained from similar conspecific populations of *A. simplex* (s. s.) analysed at other nuclear markers (allozymes) (Mattiucci *et al.*, 1997), and at mitochondrial gene level (mtDNA cox2) (Mattiucci *et al.*, 2018b).

Further, the F_{IT} value higher than zero, as found in both *A. pegreffii* and *A. simplex* (s. s.) (Suppl. Table 1a and b), seems to indicate that a certain subdivision between infrapopulations of parasites from different hosts would exist. However, the high level of polymorphism observed at the scored SSRs loci, would require a larger number of specimens to be studied in all the parasite populations collected from different host species, in order to find causes of those differences at the infrapopulation level.

Finally, higher estimates of Nei's *D* genetic distance was inferred at the interspecific level between *A. pegreffii* and *A. simplex* (s. s.) (on average, D_{Nei} = 0.91), based on five considered SSRs loci, with respect to that previously observed between the two sibling species, as estimated from allozyme loci (on average, D_{Nei} = 0.36). However, the latter value was based on genetic data inferred from a larger number of loci scored (N = 24) in populations of the two *Anisakis* species (Mattiucci *et al.*, 1997, 2014).

Concluding remarks

Among the nuclear codominant markers applied to discriminate the two species (i.e. *A. simplex* (s. s.) and *A. pegreffii*) of the *A. simplex* (s. l.) complex, so far allozymes and EF1 α-1 nDNA had provided valuable genetic data for the distinctiveness, reproductive isolation, and absence of gene flow between *A. simplex* (s. s.) and *A. pegreffii* (Mattiucci *et al.*, 2014, 2018a, 2018b). In the present study, a new set of nuclear markers (SSRs markers) was selected and validated in the two *Anisakis species*. These novel markers clearly permit to further confirm that *A. simplex* (s. s.) and *A. pegreffii* are two distinct species. This finding also remarks the utility of the detected SSRs in the identification of the two species, since SSRs achieve a high-discriminatory power in a nuclear multilocus genotyping approach.

Moreover, the latter approach would provide a powerful mean to investigate, in future researches, various microevolutionary aspects in anisakid taxa, as well as the detection of possible patterns of hybridization/introgression events between the two species in sympatric areas (Mattiucci *et al*., 2018a; and pers.com.). Thus, the developed SSRs loci will be applicable for studies of *A. simplex* (s. s.) and *A. pegreffii* from other globally distributed oceanographic waters, where they are infecting several other host species. Furthermore, they will also permit to infer data on the genetic structure of other genetically closely related species, such as *A. berlandi*, through cross-amplification studies.

SSRs markers have enabled to investigate the genetic variability and population genetic structure of *A. pegreffii* and *A. simplex* (s. s.). High genetic variability and low level of population genetic structure was generally found in the two species. This finding would be the result of a large effective population size generally observed in these parasites species, at both intermediate/paratenic (fish) and definitive (cetaceans) hosts level, and the high level of gene flow between populations of the two species, maintained by the large migration habits of

some hosts through geographically distant basin waters. The differentiation observed among populations of the two species was generally low. Values found among Boreal and Austral populations within the species *A. pegreffii* could be explained by smaller population size which could characterize the population of *A. pegreffii* from the Mediterranean Sea, with respect to that from the Austral Region; however, further genetic analysis and data on the parasite population density of this parasite species are required to support this hypothesis.

Finally, the SSRs markers could be potentially used for wider and advanced population genetics analyses, to investigate a genetic background of parasite adaptation to different host species, and to monitor the genetic variability values of populations of those parasite species. This last aspect would represent a further tool to investigate the possible use of *Anisakis* species and their genetic diversity at nuclear level, as ecological indicators of the impact of anthropogenic events and of global climate changes, providing insights on the stability of trophic webs of marine ecosystems, as previously demonstrated in anisakids on the basis of other nuclear markers (Mattiucci and Nascetti, 2007, 2008).

As it has been recently outlined (Cole and Viney, 2018), new methods of analysing population genetics of parasitic nematodes will be available in future studies, such as those gathered from whole-genome sequencing analysis and (dd)RADSeq. Thus, our understanding of nematodes population genetics, biology, evolutionary history and how they are able to respond to selective pressure will dramatically increase. This will likely help to provide a wider comprehensive picture of the ecological and epidemiological drivers (Mattiucci *et al*., 2018a) shaping the population genetic structure of the species of the *A. simplex* (s. l.) complex.

2.5 TABLES AND FIGURES

Table 1. Number of specimens of *A. pegreffii* and *A. simplex* (s. s.) analysed at the eight microsatellite loci and EF1 α-1 (nDNA) gene, reported with host species and sampling area. $N_A♂♂$, number of adult male nematodes, $N_A♀♀$, number of adult female nematodes; N_{L4} and N_{L3}, number of specimens at fourth and third larval stages, respectively.

Parasite species	Sampling area	Code area	Host species	N	N_A ♂♂	N_A ♀♀	N_{L4}	N_{L3}	7 SSRs-DNA loci (see Table 2)	*AnisL7* SSRs-DNA	EF1 α-1 nDNA
Anisakis pegreffii	SW Pacific Ocean New Zealand coast (44° 30'S - 172° 58'E)	NZ	*Cephalorhynchus hectori*	3	1	2	-	-	3	3	3
			Globicephala melas	158	36	50	57	-	158	135	158
			Grampus griseus	3	-	-	3	-	3	2	3
	SW Atlantic Ocean Argentina coast (41° 30'S - 64° 15'W)	AR	*Macruronus magellanicus*	46	-	-	-	46	46	45	46
			Merluccius hubbsi	31	-	-	-	31	31	31	31
	Mediterranean Sea Tyrrhenian Sea (41°7'N – 13°24'E)	TY	*Engraulis encrasicolus*	1	-	-	-	1	1	1	1
			Lepidopus caudatus	29	-	-	-	29	29	28	29
			Lophius budegassa	2	-	-	-	2	2	2	2
			Merluccius	39	-	-	-	39	39	33	39
			Scomber scombrus	13	-	-	-	13	13	10	13
	Western Adriatic Sea (42°18'N – 15°35'E)	AD	*Stenella coeruleoalba*	58	22	25	11	-	58	45	58
			Lophius piscatorius	3	-	-	-	3	3	3	3
			Merluccius	38	-	-	-	38	38	27	38
			Scomber scombrus	7	-	-	-	7	7	5	7
			Trachurus	20	-	-	-	20	20	2	20
				Tot 451					*451*	*372*	*451*
Anisakis simplex (s. s.)	NE Atlantic Ocean Gran Sole Bank (49° 38'N–10° 10'W)	GS	*Merluccius*	115	-	-	-	115	115	70	115
	English Channel (48° 38' N -4° 34' W)	EC	*Phocoena*	79	45	34	-	-	79	77	79
			Clupea harengus	97	-	-	-	97	97	54	97
	North Sea (59° 13'N–00° 14'W)	NS	*Clupea harengus*	67	-	-	-	67	67	50	67
	Norwegian Sea (68° 52'N–3° 08'E)	NW	*Clupea harengus*	41	-	-	-	41	41	39	41
			Micromesistius poutassou	10	-	-	-	10	10	-	10
			Scomber scombrus	26	-	-	-	26	26	8	26
	Baltic Sea (58° 29'N–19° 51'E)	BA	*Clupea harengus*	57	-	-	-	57	57	43	57
				Tot 492					*492*	*341*	*492*

Table 2. Locus name, primer sequences, number of alleles (N_A), repeat type, fragment bp size and fluorescent dye of seven microsatellite loci developed in *A. pegreffii*, which cross amplify in *A. simplex* (s. s.). *=labeled primer.

Locus	Primer sequence 5' – 3'	N_A	Repeat type	Range size (bp)	Dye
Multiplex 1					
Anisl 05784	F: GGGTTTGGACACTGGTTTGG* R: TGCAATCGTCATTTCTGCCTC	17	(TGT) 11	69 - 91	VIC (green)
Anisl 08059	F: CCCTTCTCTCTGTGGAGTCG* R: TGCTGCTATTCGAGCGTTTG	15	(CATC) 4	80 - 124	PET (red)
Anisl 00875	F: TGACGCTCGAGTTGGTACAG* R: GGTGGTGATGTTTACGCGAC	12	(GCA) 8	147 - 159	NED (yellow)
Anisl 07132	F: ATCAGTGCCGAGTAGCATGG* R: TTCAGGGTGCAAATGACGTG	12	(ATTG) 7	216 - 250	FAM (blue)
Multiplex 2					
Anisl 00314	F: CGTAGTGCTTCGCTTATCGC* R: AGGGGATATGATCGAGATTAGACAG	14	(GATA) 7	90 - 106	VIC (green)
Anisl 10535	F: GTTTTGGGTTACCACCGACC* R: GCAATGGGCAGTCATGGAAG	9	(TTG) 9	130 - 143	PET (red)
Anisl 00185	F: CCGTGAACGCGATTCTCAAC* R: CCGCCTCCAAACAAACAAAC	10	(TTG) 7	182 - 204	FAM (blue)

Table 3. Genetic diversity at eight microsatellite loci of adult and larval specimens in nine populations of *A. pegreffii* and *A. simplex (*s. s.) analysed in the present study. For population code, see Table 1. *N*, total number of genotyped nematodes at each locus; *Ho*,observed heterozygosity; *He*, expected heterozygosity; *A*, number of alleles detected at each locus; *U*, unique alleles, occurring at frequency >0.1, among the populations of the two species. *P*, indicates the significance (P<0.05) of the deviation from HWE expectation. ****P*<<0.001, **P*<0.01.

		A. pegreffii				*A. simplex* (s. s.)				
Locus		NZ	AR	TY	AD	GS	EC	NS	NW	BA
Anisl00185	N	164	77	84	126	115	176	67	77	57
	Ho	0.77	0.91	0.73	0.68	0.55	0.51	0.64	0.46	0.58
	He	0.82	0.84	0.80	0.78	0.72	0.70	0.75	0.74	0.76
	P-value	0.22	0.59	0.35	0.26	*	*	0.01	*	0.01
	A (U)	9(0)	8(0)	9(0)	8(0)	8(0)	8(0)	8(0)	6(0)	8(0)
Anisl00314	N	164	77	84	126	115	176	67	77	57
	Ho	0.46	0.34	0.38	0.49	0.22	0.11	0.13	0.21	0.18
	He	0.76	0.72	0.74	0.77	0.82	0.77	0.85	0.80	0.80
	P-value	***	***	***	***	***	***	***	***	***
	A (U)	9(0)	7(0)	6(0)	8(0)	9(0)	13(2)	9(0)	10	8(1)
Anisl10535	N	164	77	84	126	115	176	67	77	57
	Ho	0.71	0.75	0.44	0.29	0.13	0.14	0.19	0.12	0.16
	He	0.76	0.76	0.43	0.34	0.13	0.14	0.18	0.11	0.15
	P-value	0.41	0.86	0.54	0.26	1.00	1.00	1.00	1.00	1.00
	A (U)	9(1)	7(0)	6(0)	7(0)	5(0)	5(0)	4(0)	5(0)	3(0)
Anisl07132	N	164	77	84	126	115	176	67	77	57
	Ho	0.69	0.70	0.73	0.75	0.66	0.68	0.60	0.68	0.68
	He	0.76	0.68	0.73	0.82	0.74	0.78	0.71	0.74	0.68
	P-value	0.06	0.71	0.48	0.06	0.06	0.15	0.06	0.06	0.19
	A (U)	9(0)	10(0)	9(0)	10(0)	11(0)	12(1)	11(0)	10(0)	7(0)
Anisl05784	N	164	77	84	126	115	176	67	77	57
	Ho	0.81	0.71	0.82	0.83	0.66	0.62	0.61	0.60	0.70
	He	0.80	0.76	0.78	0.81	0.73	0.69	0.71	0.71	0.74
	P-value	0.38	0.06	0.89	0.62	0.69	0.10	0.10	0.06	0.23
	A (U)	13(0)	10(0)	8(1)	11(0)	12(0)	13(0)	11(0)	10(0)	9(0)
Anisl08059	N	164	77	84	126	115	176	67	77	57
	Ho	0.65	0.78	0.63	0.69	0.26	0.17	0.20	0.14	0.30
	He	0.83	0.81	0.84	0.84	0.28	0.21	0.27	0.14	0.30
	P-value	0.06	0.43	0.05	0.07	0.23	0.06	0.06	1.00	1.00
	A (U)	11(0)	12(2)	11(0)	11(1)	7(0)	6(0)	4(0)	6(1)	5(0)
Anisl00875	N	164	77	84	126	115	176	67	77	57
	Ho	0.42	0.53	0.52	0.51	0.75	0.63	0.54	0.46	0.74
	He	0.45	0.51	0.52	0.51	0.70	0.67	0.62	0.61	0.66
	P-value	0.34	0.63	0.19	0.76	0.75	0.06	0.29	0.06	0.77
	A (U)	8(0)	9(1)	6(0)	9(0)	10(0)	8(0)	9(0)	8(0)	9(0)
Anisl7	N	140	76	74	82	70	131	50	47	43
	Ho	0.21	0.04	0.24	0.29	0.40	0.32	0.18	0.38	0.21
	He	0.25	0.14	0.53	0.47	0.65	0.70	0.65	0.61	0.70
	P-value	***	***	***	***	***	***	***	***	***
	A (U)	4(1)	3(0)	3(0)	2(0)	9(1)	9(2)	8(1)	8(0)	8(0)

Table 4. Observed (H_o) and expected (H_e) heterozigosity at the two sex-linked loci (i.e. Anis00314 and Anisl7) in A. pegreffii and A. simplex (s. s.), estimated in adult female specimens. N= number of individuals genotyped at those loci. For the population codes, see Table 1. P= indicates the significance (P<0.05) of the deviation from HWE expectation. **P<0.01*, ***P<0.0001*, ****P<<0.0001*.

		A. pegreffii		*A. simplex* (s. s.)
Locus		NZ ♀♀	AD ♀♀	EC ♀♀
Anisl 00314	*N*	52	25	34
	Ho	0.67	0.84	0.24
	He	0.77	0.77	0.80
	p-value	n.s.	n.s.	***
Anisl 7	*N*	52	25	34
	Ho	0.19	0.67	0.67
	He	0.21	0.48	0.75
	p-value	n.s.	n.s.	n.s.

Table 5. Allele frequencies observed at six microsatellite loci tested in the nine populations of *A. pegreffii* and *A. simplex* (s. s.). For population codes, see Table 1. With regard to the sex-linked locus (i.e. *Anisl 7*) the most reliable estimate of allele frequencies was calculated only on adult populations of the two species, 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. Populations of the two species including only larval specimens (see Table 1) tested at the sex-linked locus *Anisl 7*, were excluded from the allele frequencies estimate.

		Anisakis pegreffii				*A simplex* (s. s.)				
Population Code		NZ	AR	TY	AD	GS	EC	NS	NW	BA
Locus										
Anisl 10535	N	164	77	84	126	115	176	67	77	57
	Allele									
	125	0.01	-	-	-	-	0.01	-	0.01	-
	128	0.01	0.01	-	0.01	0.01	-	-	0.01	-
	131	0.01	0.02	0.04	0.01	0.02	0.02	0.02	0.01	-
	134	0.19	0.14	0.04	0.01	0.93	0.93	0.90	0.94	0.92
	137	0.20	0.28	0.07	0.05	0.03	0.03	0.07	0.03	0.07
	140	0.26	0.26	0.73	0.81	0.01	0.01	0.01	-	0.01
	143	0.30	0.27	0.10	0.09	-	-	-	-	-
	146	0.01	0.02	0.02	0.02	-	-	-	-	-
	149	0.01	-	-	-	-	-	-	-	-
Locus										
Anisl 07132	N	164	77	84	126	115	176	67	77	57
	Allele									
	208	-	0.01	-	-	0.03	0.02	0.01	0.03	-
	212	0.05	0.03	0.04	0.02	0.05	0.05	0.04	0.04	0.02
	216	0.14	0.13	0.13	0.16	0.03	0.04	0.04	0.03	0.04
	220	0.42	0.53	0.46	0.32	0.30	0.30	0.17	0.21	0.46
	224	0.15	0.09	0.14	0.17	0.39	0.34	0.49	0.45	0.31
	228	0.08	0.10	0.11	0.09	0.12	0.08	0.07	0.11	0.12
	232	0.08	0.06	0.04	0.11	0.04	0.08	0.09	0.06	0.04
	236	0.04	0.03	0.06	0.06	0.01	0.04	0.05	0.04	-
	240	0.03	0.01	0.01	0.04	0.01	0.02	0.02	0.01	0.01
	244	-	-	-	0.02	0.01	0.01	0.01	-	-
	248	0.01	0.01	0.01	-	-	0.01	0.01	0.02	-
	252	-	-	-	-	-	0.01	-	-	-
	256	-	-	-	0.01	0.01	-	-	-	-
Anisl 05784	N	164	77	84	126	115	176	67	77	57
	Allele									
	057	-	-	-	-	0.01	0.01	-	-	-
	060	-	-	-	-	0.01	0.01	0.01	-	-
	063	-	0.01	-	-	0.28	0.19	0.12	0.24	0.22
	066	0.01	-	-	-	0.04	0.01	0.08	0.05	0.03
	069	0.01	-	-	-	0.03	0.01	0.04	0.02	0.05
	072	0.01	-	-	0.01	0.02	0.02	0.01	0.01	0.01
	075	0.01	0.03	-	0.01	0.02	0.03	0.03	0.01	0.03
	078	0.07	0.02	-	0.02	0.41	0.50	0.49	0.45	0.45
	081	0.03	0.03	0.08	0.06	0.11	0.12	0.15	0.17	0.11
	084	0.08	0.06	0.06	0.08	0.05	0.07	0.05	0.03	0.07
	087	0.24	0.28	0.21	0.17	0.01	0.01	0.01	0.01	0.03
	090	0.32	0.37	0.30	0.30	0.01	0.01	0.01	0.01	-
	093	0.16	0.14	0.23	0.22	-	-	-	-	-
	096	0.04	0.05	0.07	0.06	-	0.01	-	-	-
	099	0.01	0.01	0.04	0.06	-	-	-	-	-
	102	0.01	-	-	0.01	-	-	-	-	-
	105	-	-	0.01	-	-	-	-	-	-

(continues)

(continued)

		Anisakis pegreffii				*A simplex* (s. s.)				
Population Code		NZ	AR	TY	AD	GS	EC	NS	NW	BA
Locus										
Anisl 08059	N	164	77	84	126	115	176	67	77	57
	Allele									
	078	-	-	-	-	-	-	-	0.01	-
	082	0.02	-	0.02	0.01	0.03	0.01	0.04	0.01	0.04
	086	0.27	0.15	0.30	0.26	0.84	0.89	0.85	0.93	0.83
	090	0.03	0.01	0.04	0.04	0.06	0.05	0.10	0.03	0.08
	094	0.12	0.07	0.12	0.19	0.01	-	-	-	-
	098	0.11	0.08	0.17	0.16	0.04	0.03	0.01	0.01	0.03
	102	0.21	0.36	0.08	0.10	0.01	0.01	-	-	-
	106	0.13	0.12	0.14	0.11	-	0.01	-	-	-
	110	0.05	0.12	0.05	0.04	0.01	-	-	0.01	-
	114	0.04	0.03	0.04	0.06	-	-	-	-	-
	118	0.01	0.03	0.03	0.02	-	-	-	-	-
	122	-	-	-	0.01	-	-	-	-	-
	126	0.01	0.01	0.01	-	-	-	-	-	-
	130	-	0.01	-	-	-	-	-	-	-
	134	-	0.01	-	-	-	-	-	-	-
Anisl 00875	N	164	77	84	126	115	176	67	77	57
	Allele									
	142	.	.	.	.	0.01	-	-	0.01	0.01
	145	0.01	0.01	0.01	0.01	0.01	-	0.01	-	0.01
	148	0.02	-	-	0.01	0.01	0.01	0.01	0.01	0.01
	151	0.03	0.06	0.05	0.05	0.02	0.02	0.02	0.02	-
	154	0.02	0.02	0.04	0.04	0.03	0.02	0.01	0.01	0.03
	157	0.72	0.67	0.67	0.68	0.17	0.21	0.20	0.23	0.17
	160	0.15	0.17	0.16	0.16	0.48	0.51	0.57	0.56	0.53
	163	0.04	0.04	0.07	0.03	0.20	0.12	0.14	0.15	0.18
	166	0.01	0.01	-	0.01	0.05	0.08	0.03	0.01	0.05
	169	-	-	-	-	0.02	0.03	0.01	-	-
	172	-	0.01	-	0.01	-	-	-	-	0.01
	175	-	0.01	-	-	-	-	-	-	-
Anisl 7	N	80	/	/	34	/	59	/	/	/
	Allele									
	216	0.01	-	-	-	-	-	-	-	-
	219	0.05	-	-	0.39	-	-	-	-	-
	222	0.90	-	-	0.61	-	-	-	-	-
	225	0.04	-	-	-	-	-	-	-	-
	246	-	-	-	-	-	-	-	-	-
	249	-	-	-	-	-	-	-	-	-
	252	-	-	-	-	-	0.13	-	-	-
	255	-	-	-	-	-	0.48	-	-	-
	258	-	-	-	-	-	0.18	-	-	-
	261	-	-	-	-	-	0.11	-	-	-
	264	-	-	-	-	-	0.04	-	-	-
	267	-	-	-	-	-	0.03	-	-	-
	270	-	-	-	-	-	0.02	-	-	-
	273	-	-	-	-	-	-	-	-	-
	276	-	-	-	-	-	-	-	-	-
	279	-	-	-	-	-	-	-	-	-
	285	-	-	-	-	-	0.01	-	-	-

Table 6. Population pairwise F_{st} (below the diagonal) estimates by FSTAT (Goudet, 1995), and Nei's genetic distance (Nei, 1978) values (D_{Nei}, above the diagonal) calculated by BIOSYS.2 (Swofford and Selander, 1997) from the allele frequencies observed at five SSRs loci (excluding *Anisl 00185*, *Anisl 00314* and *Anisl 7*), among the nine analyzed populations of *A. pegreffii* (NZ: New Zealand coast; AR: Argentine coast; TY: Tyrrhenian Sea; AD: Adriatic Sea), and *A. simplex* (s. s.) (GS: Grand Sole Bank; EC: English Channel; NS: North Sea; NW: Norwegian Sea; BA: Baltic Sea). Significant genetic differentiation: * P<0.01; ** *P<0.001*, based on 1000 permutations.

	NZ	AR	TY	AD	GS	EC	NS	NW	BA
NZ	-	0.029	0.107	0.144	0.721	0.676	0.742	0.735	0.670
AR	0.007*	-	0.148	0.200	0.942	0.883	1.006	0.990	0.845
TY	0.046**	0.060**	-	0.005	0.965	0.891	1.006	0.990	0.904
AD	0.058**	0.077**	0.002	-	1.084	1.010	1.089	1.094	1.049
GS	0.243**	0.293**	0.311**	0.318**	-	0.030	0.012	0.004	0.004
EC	0.254**	0.308**	0.325**	0.330**	0.003	-	0.049	0.034	0.038
NS	0.243**	0.297**	0.315**	0.317**	0.011*	0.008	-	0.002	0.023
NW	0.257**	0.315**	0.331**	0.333**	0.004	0.004	.002	-	0.017
BA	0.226**	0.271**	0.296**	0.307**	0.003	0.004	.021*	0.016*	-

Table 7. Example of a molecular key for the identification of specimens of *A. pegreffii* and *A. simplex* (s. s.), based on the alleles detected at four SSRs loci (i.e. *Anisl 05784, Anisl 08059, Anisl 10535, Anisl 7*) (see Table 5), to be combinable in a single one multiplex PCR reaction.

	Locus	Alleles		Species
1.	*Anisl 05784*	*93, 99, 102, 105*	→	*A. pegreffii*
		57, 60	→	*A. simplex* (s. s.)
		78, 63, 90, 87, 81, 84, 66, 96, 69, 75. 72		2.
2.	*Anisl 08059*	*114, 118, 122, 126, 130, 134*	→	*A. pegreffii*
		78	→	*A. simplex* (s. s.)
		86, 102, 106, 94, 110, 90, 114, 118		3.
3.	*Anisl 10535*	*143, 146, 149*	→	*A. pegreffii*
		125	→	*A. simplex* (s. s.)
		140, 137, 134, 131, 128		4.
4.	*Anisl 7*	*216, 219, 222, 225*	→	*A. pegreffii*
		246, 249*, 252, 255, 258, 261, 264, 267, 270*	→	*A. simplex* (s. s.)
		273, 276*, 279*, 285*		

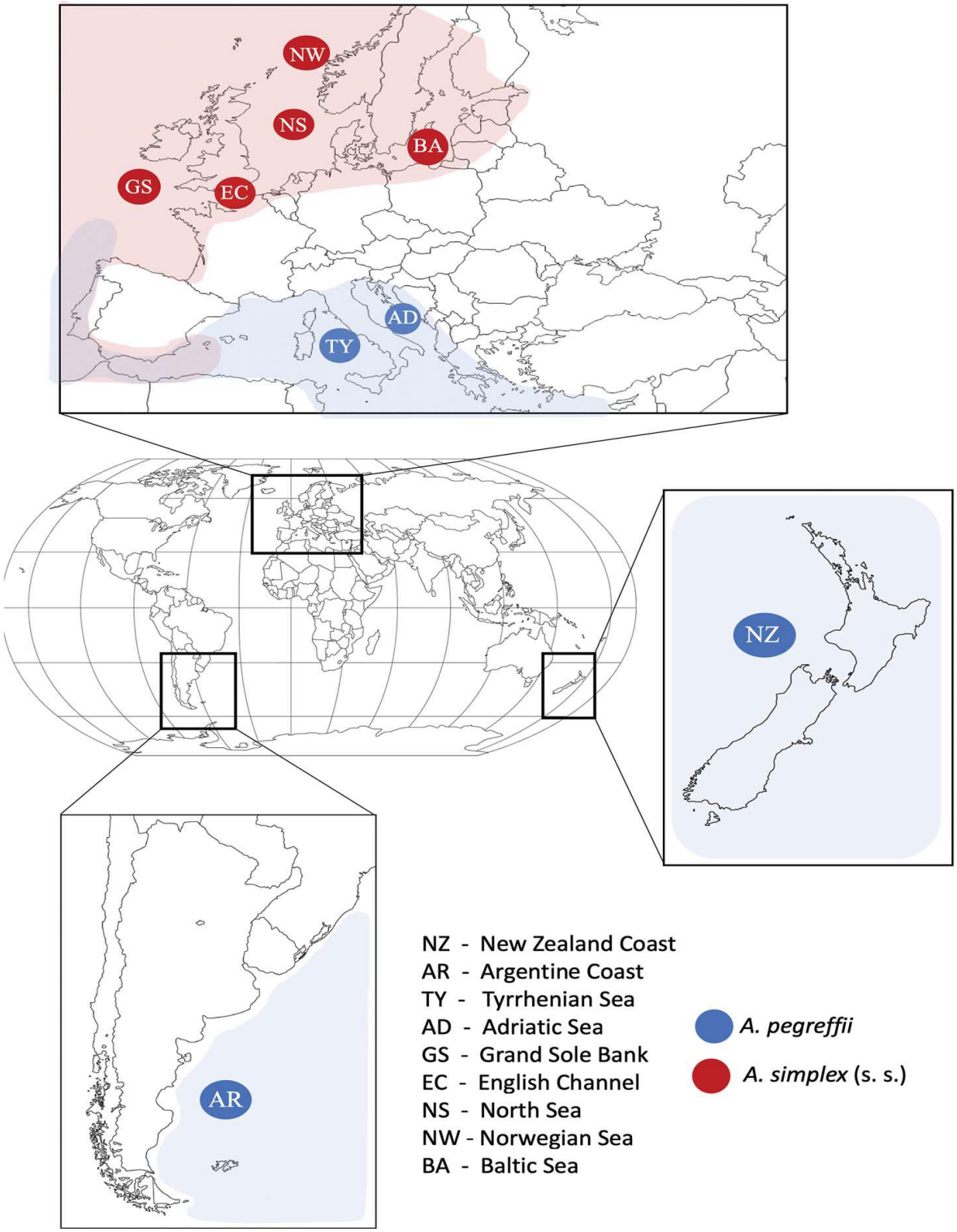


Figure 1: Collecting sites of populations of *A. simplex* (s. s.) and *A. pegreffii* analysed in the present study, mapped into the geographical range of the two parasites species (for location reference codes see Table 1).

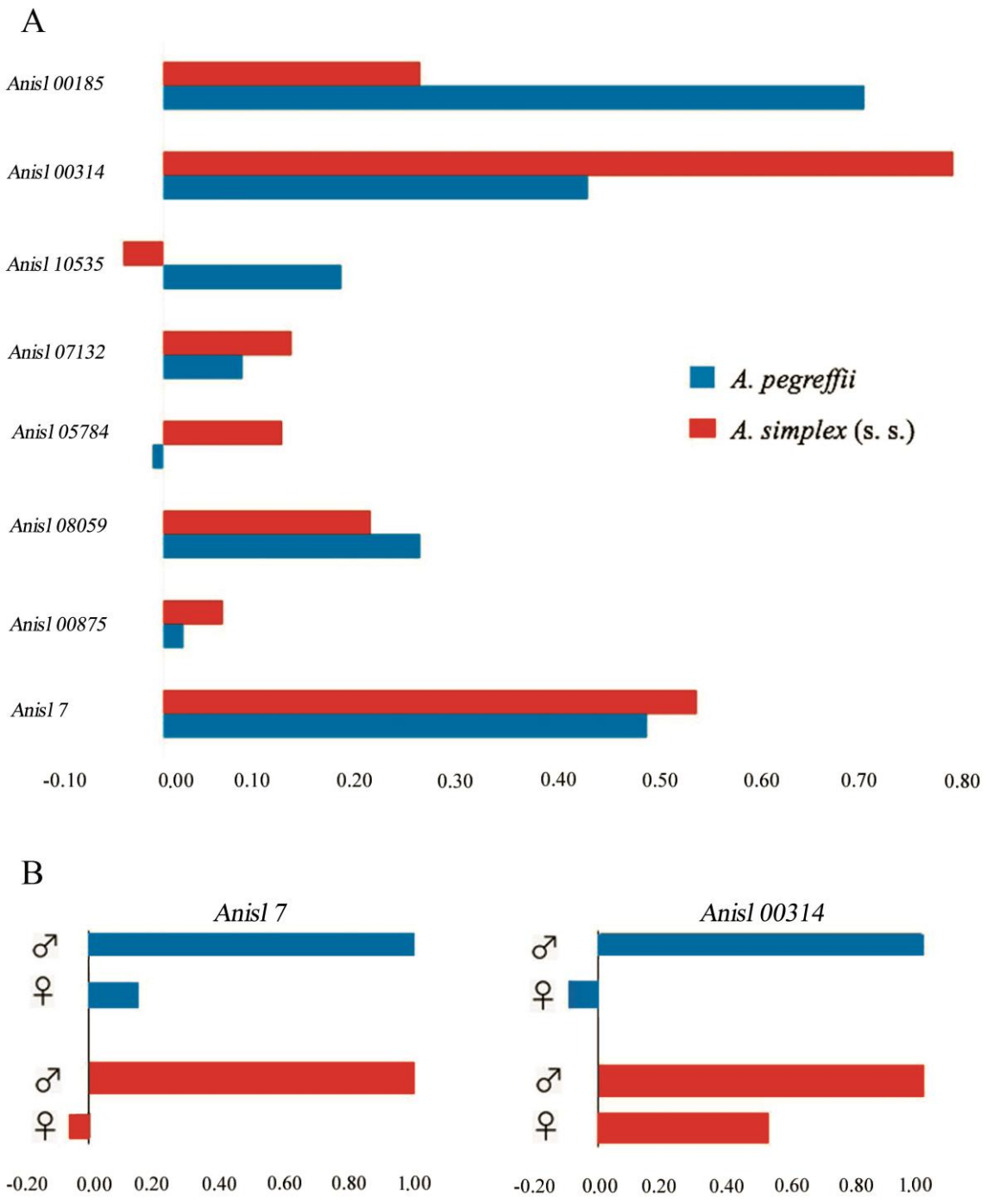


Figure 2: (A) F_{IS} calculated at the eight microsatellites loci studied in the two species *A. pegreffii* and *A. simplex* (s. s.). Negative values indicate heterozygous excess while positive values indicate homozygous excess from that expected under Hardy–Weinberg Equilibrium (HWE) and (B) F_{IS} in male and female specimens of *A. pegreffii* and *A. simplex* (s. s.), at the two sex-linked loci, i.e. *Anisl 00314* and *Anisl 7*.

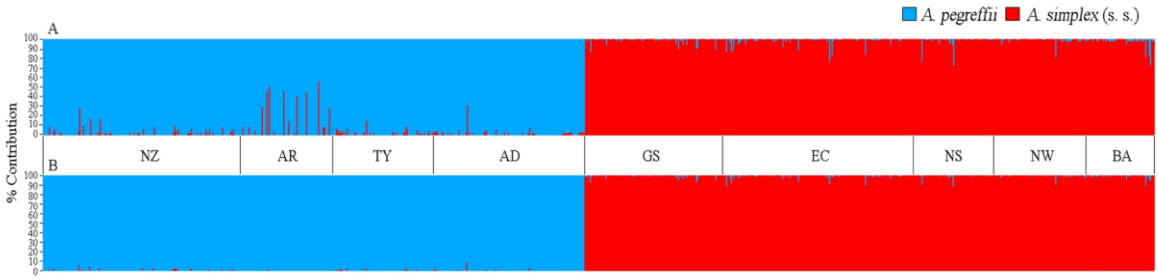


Figure 3: Percentage contribution (Q value) of *A. pegreffii* and *A. simplex* (s. s.) to the multi-locus genotype of each studied individual (barplot), estimated at five SSRs loci (i.e. *Anisl 10535*, *Anisl 05784*, *Anisl 00875*, *Anisl 07132* and *Anisl 08059*), among those here studied (A); and at the same five loci plus EF1 α-1 (B), by using STRUCTURE, with k = 2. Codes of the sampling areas (see Fig. 1 and Table 1) are as follows: NW: Norwegian Sea; NS: North Sea; GS: Grand Sole bank; EC: English Channel; BA: Baltic Sea; AD: Adriatic Sea; TY: Tyrrhenian Sea; NZ: New Zealand coast and AR: Argentine coast.

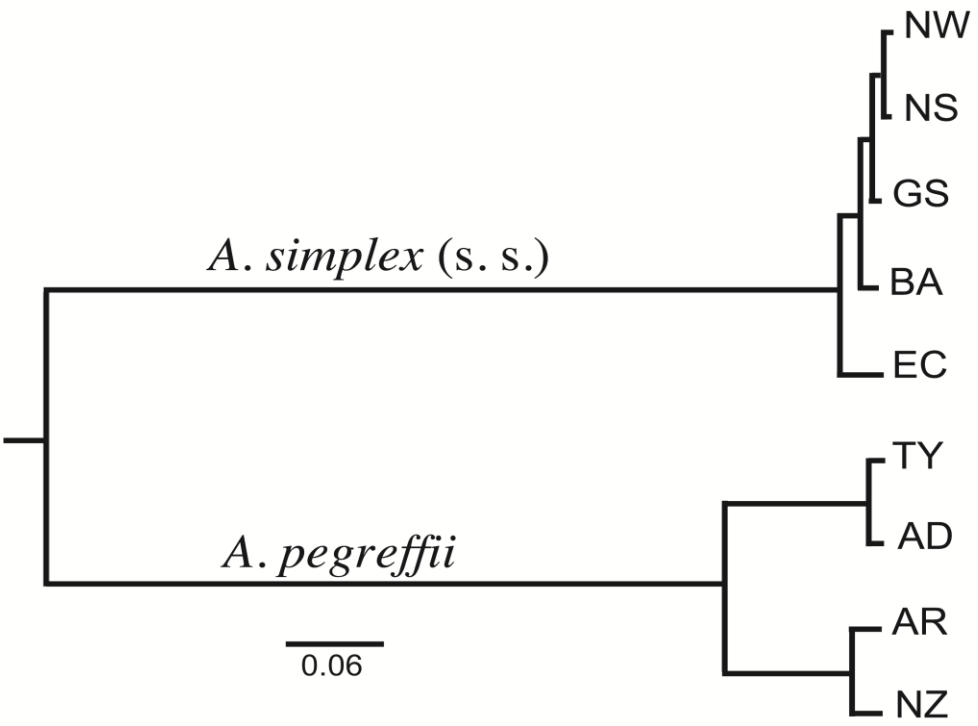


Figure 4: The unweighted pair group method of analysis (UPGMA) cluster based on Nei's genetic distance values inferred from allelic frequencies calculated at five microsatellite loci (i.e. *Anisl 10535, Anisl 05784, Anisl 00875, Anisl 07132, Anisl 08059*), showing the genetic relationship between populations and species here studied (*A. pegreffii* and *A. simplex* (s.s.)). Populations and their reference codes are reported in Table 1 and Fig. 1. They are: NW: Norwegian Sea; NS: North Sea; GS: Grand Sole bank; EC: English Channel; BA: Baltic Sea; AD: Adriatic Sea; TY: Tyrrhenian Sea; NZ: New Zealand coast and AR: Argentine coast.

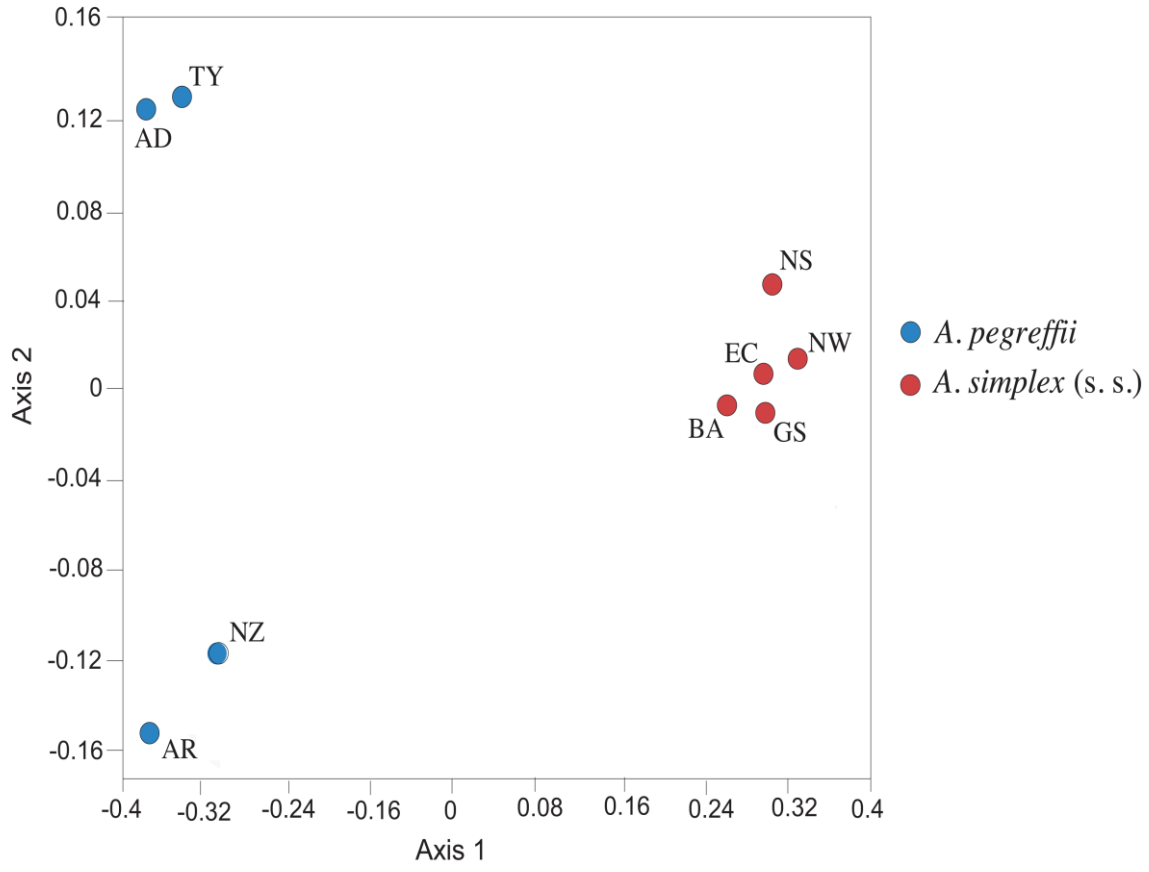


Figure 5: Non-metric multidimensional scaling (nMDS) ordination plot inferred from Euclidean distance values obtained from the allele frequencies observed at five microsatellite loci (i.e. *Anisl 10535*, *Anisl 05784*, *Anisl 00875*, *Anisl 07132*, *Anisl 08059*), showing the genetic relationships of populations and species here studied (i.e. *A. pegreffii* and *A. simplex* (s.s.)). The nMDS-plot for the data set was performed by Vegan Package. R package version 2.5-4 (Oksanen *et al.*, 2019). The variance percentage explained by the two axes is the following: 47.7% for axis_1 and 7.1% for axis_2; stress value = 0.0003. Populations and their reference codes are reported in Table 1 and Fig. 1. They are: EC: English Channel; GS: Grand Sole bank; NW: Norwegian Sea; NS: North Sea; BA: Baltic Sea; AD: Adriatic Sea; TY: Tyrrhenian Sea; NZ: New Zealand coast and AR: Argentine coast.

Supplementary Table 1. Analysis of molecular variance (AMOVA) between and within populations of the two species *A. pegreffii* (a) and *A. simplex* (s. s.) (b), using genetic data sets from five SSRs loci (*Anisl 10535*, *Anisl 05784*, *Anisl 00875*, *Anisl 08059* and *Anisl 07132*). The *P* values to the fixation indices are reported as ***$p<0.001$, * $p<0.05$.

a)

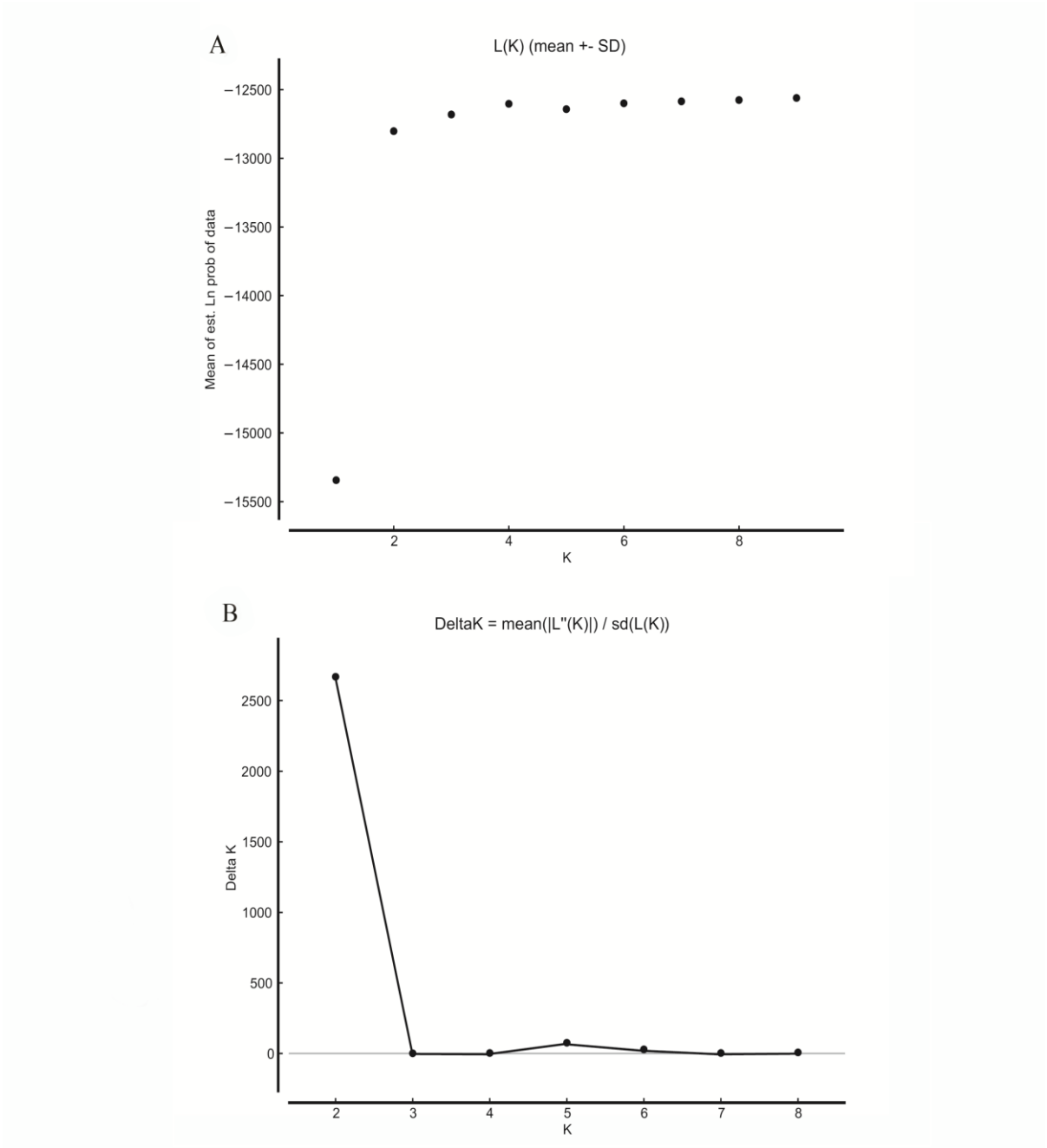
Anisakis pegreffii

Source of variation	d.f.	Sum of squares	Variance components	% of variation	Fixation indices
among populations	3	55.373	0.076	4.21	FST= 0.042 ***
Among individuals within populations	447	837.778	0.146	8.10	FIS= 0.084 ***
within individuals	451	713.500	1.582	87.69	FIT= 0.123 ***
total	901	1606.651	1.804		

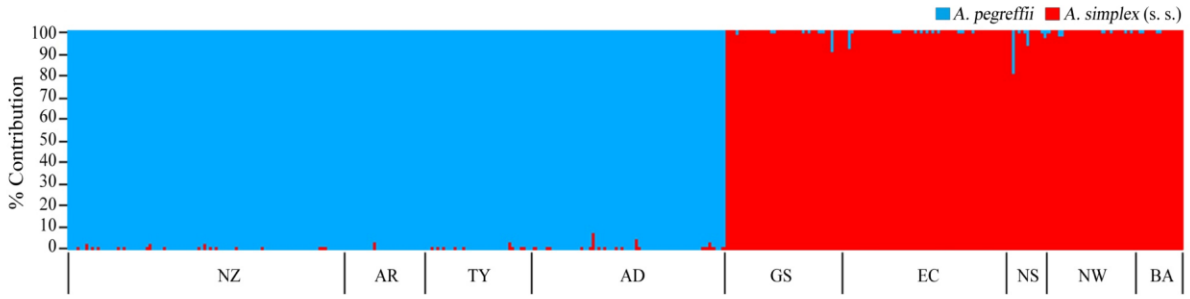
b)

Anisakis simplex (s. s.)

Source of variation	d.f.	Sum of squares	Variance components	% of variation	Fixation indices
among populations	4	10.598	0.007	0.56	FST= 0.006*
Among individuals within populations	470	641.632	0.124	9.95	FIS= 0.100 ***
within individuals	475	530.500	1.117	89.49	FIT= 0.105 ***
total	949	1182.729	1.248		



Supplementaru Figure 1: Estimate of the best number of clusters (K) describing the population structure of the Anisakis spp. samples analyzed in the present study. The computation was carried out by using STRUCTURE HARVESTER: A) highes Ln-probability and B) DeltaK optimality criteria.



Supplementary Figure 2: shows the percentage contribution (Q value) of *Anisakis pegreffii* and *A. simplex* (s. s.) to the multi-locus genotype of each studied individual (barplot), (only considering the genotypes of adult female specimens) here estimated at the six SSR loci (including *Anisl 7*, but excluding *Anisl 00185* and *Anisl 00314*), by using STRUCTURE with k=2. Codes of the Anisakis spp. populations are those detailed in Fig.1 and Table 1.

3. STUDY 2

CROSS-SPECIES UTILITY OF MICROSATELLITE LOCI FOR THE GENETIC CHARACTERISATION OF *ANISAKIS BERLANDI* (NEMATODA: ANISAKIDAE)

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Abstract

Eight microsatellite loci, recently developed in the species *Anisakis pegreffii*, have here successfully amplified in *Anisakis berlandi*, sibling species of the *A. simplex* (s. l.) complex. They were validated on adult specimens (N= 46) of the parasite species, collected from two individuals of the definitive host, the long-finned pilot whale *Globicephala melas* from New Zealand waters. Among the eight loci scored, one has shown null alleles in *A. berlandi*: i.e. *Anisl 07132*; thus, it was excluded from the subsequent genetic analysis. Two loci were monomorphic: i.e. *Anisl 00314* and *Anisl 10535*. In addition, as also previously detected in the other species of the *A. simplex* (s. l.) complex, the locus *Anisl 7* was seen to be sex-linked, showing hemizygosity in male specimens. Differential allele frequency distributions of *A. berlandi*, with respect to those previously observed in *A. pegreffii* and *A. simplex* (s. s.), were found at some microsatellite loci. The locus *Anisl 7* was found 100% diagnostic between *A. berlandi* and *A. pegreffii*; while others resulted 99% diagnostic between *A. berlandi* and the other two species. SSRs loci allowed also to estimate genetic differentiation of *A. berlandi* from *A. pegreffii* (*Fst* ≈ 0.45, Dc= 0.82) and *A. simplex* (s. s.) (*Fst* ≈ 0.57, *Dc*= 0.73). The results suggest that SSRs provide a set of candidate markers for population genetics analysis

of *A. berlandi*, as well as in the investigation, in a multi-locus genotyping approach, of possible patterns of hybridization/introgression events between *A. berlandi* and the other two *Anisakis* species in sympatric conditions.

Keywords: *Anisakis berlandi*, microsatellites, genetic diversity, nuclear markers, sex-linkage loci

3.1 INTRODUCTION

Anisakis berlandi Mattiucci, Cipriani, Webb, Paoletti, Marcer, Bellisario, Gibson, Nascetti, 2014 (Mattiucci *et al.*, 2014) is a nematode belonging to the *A. simplex* (s. l.) complex. It has been first described as *A. simplex* C (see Mattiucci *et al*., 1997), co-infecting with *A. simplex* (s. s.), at adult stage, the false killer whale *Pseudorca crassidens*, in Pacific Ocean waters (Vancouver Island) and genetically recognised, as third stage larva, in fish species from off New Zealand waters (Mattiucci *et al*., 1997). Later, the nomenclatural designation and formal description for *A. simplex* species C with the name *A. berlandi* was given (Mattiucci *et al.*, 2014). Key morphological diagnostic traits between *A. berlandi* and the other species of the *A. simplex* complex (i.e. *A. pegreffii* and *A. simplex* (s. s.)) were also proposed (Mattiucci *et al.*, 2014). A procrustes analysis, combining both morphological and genetic datasets on specimens belonging to the three sibling species, showed their clustering into three well-defined groups, corresponding to the three taxa (Mattiucci *et al*., 2014). Further, a concatenated phylogenetic inference, combining both mitochondrial and nuclear sequences datasets, showed the existence of the 3 species of the *A. simplex* (s. l.) complex, as distinct phylogenetic lineages (Mattiucci *et al.*, 2014, 2018a).

Ecological data pertaining to the geographical range and host distribution revealed for *A. berlandi* a discontinuous range of distribution. This includes the Austral region: the Chilean Pacific, the South Shetland Islands, New Zealand and Australian waters, and the South African Atlantic coast (Klimpel *et al.*, 2010; Mattiucci *et al.*, 1997, 2014, 2018a; Mattiucci and Nascetti, 2008; Shamsi *et al.*, 2012, 2017). This species has been identified, at the adult stage, in sympatry and syntopy with *A. pegreffii*, in *Globicephala melas* and *Grampus gryseus* from the New Zealand, and in *G. melas* from south west (South African coast) and south east (Chilean coast) Pacific waters (Mattiucci *et al*., 2018a). Larval stages of

A. berlandi have been identified in nine fish species from Austral waters off New Zealand (Mattiucci *et al.,* 2018a), the South African coast (Mattiucci *et al.,* 2018a), Southern Shetland Islands (Klimpel *et al.,* 2010), the Southern Chilean coast, and in some unusual hosts from the New Caledonian waters (Shamsi *et al*., 2017). Few larval specimens of *A. berlandi* and *A. pegreffii* were reported in myctophids from the southern waters of the Southern Ocean (i.e. South Shetland Islands, Antarctic area) and that could be related to the introduction of those two parasites species from outside the Antarctic, through their migrating fish intermediate hosts (Klimpel *et al.,* 2010). Indeed, also the very low prevalence found in *M. leonina* from South Shetland Islands (Klimpel *et al*., 2010) could be explained as an accidental infection when that pinniped host preyed upon on infected migratory fish species (Mattiucci *et al.,* 2017). Moreover, L4 stage of *A. berlandi* were also identified in *Kogia sima* in Australian waters (Shamsi *et al.,* 2012).

The mitochondrial marker mtDNA *cox2* gene locus has been demonstrated to be informative for species recognition of all the species of the genus *Anisakis* (Mattiucci *et al.,* 2018a; Valentini *et al*., 2006), including the species *A. berlandi* (Mattiucci *et al*., 2014). While, among the nuclear markers, those inferred from allozymes, despite their utility in the discovery of anisakid species and their genetic identification at any life-history stage, are not standardized method and available in all the laboratories for species recognition. Conversely, DNA microsatellites have recently become alternative nuclear markers of choice to be used for species recognition and population genetic analysis of nematodes included in the *A. simplex* (s. l.) complex (Mattiucci *et al.,* 2019). Indeed, some DNA microsatellite loci were developed in *A. pegreffii* and *A. simplex* (s. s.) (Mladineo *et al*., 2017a); however, no diagnostic loci between those taxa were evidenced in that study. Conversely, more recently, novel DNA microsatellite loci discovered in the species *A. pegreffii* were found to cross-amplify the species *A. simplex* (s. s.) (Mattiucci *et al.,* 2019); they were also discovered to be

of diagnostic value in the recognition of *A. pegreffii* and *A. simplex* (s. s.) and for their population genetics analysis (Mattiucci *et* al., 2019).

The species *A. berlandi* was never investigated by SSRs (Simple Sequence Repeats) markers. Thus, the aim of this study was to: *(i)* validate the recent developed DNA microsatellite loci on a certain number of adult specimens of *A. berlandi*, collected from metapopulations of the parasite species included in its host range and geographical distribution*; (ii)* evaluate the genetic diversity of *A. berlandi*, as inferred from SSRs analysis; *(iii)* provide further diagnostic nuclear markers to be used in a multi-locus genetic approach allowing to distinguishing *A. berlandi* from the other two species of the *A. simplex* (s. l.) complex, i.e. *A. pegreffii* and *A. simplex* (s. s.) which can be also particularly useful to investigate possible hybridization and/or introgression events between the three sibling species; and *(iv)* estimate genetic differentiation of *A. berlandi* with respect to those *Anisakis* spp., as inferred from the SSRs.

3.2 MATERIALS AND METHODS

Parasite samples DNA extraction

Anisakis berlandi samples were collected from two individuals of the definitive host species, the long-finned pilot whale *Globicephala melas* (Traill), stranded on the New Zealand coast (44°30’S - 172°58’E). Nematodes collected from the stomach of their hosts were washed in saline solution and then preserved, frozen at -80°C, as part of the collection of anisakids stored at the Department of Public Health and Infectious Diseases of "Sapienza - University of Rome". Because of our previous findings of microsatellites among those developed, as sex-linked loci (Mattiucci *et al.,* 2019), only adult nematodes were used for the genetic characterization of the species based on SSR loci. Thus, the nematodes were first distinguished as L4-stage larvae and adults; then female and male adults were selected,

according to the main morphological features diagnostic between sexes (Mattiucci *et al*., 2014), by the use of an optical microscope at X100–400 total magnification. A total of 46 nematodes were examined from the two individual definitive hosts. The central part of each worm's body was then used for the molecular analysis, while the cephalic and caudal ends were stored for male and female discrimination. Out of the 46 adult specimens of *Anisakis* spp. detected, *N*= 24 females and *N*= 22 males were selected for the SSR analysis.

For the DNA extraction, a tissue portion of around 2mg was used from each worm specimen. The cetyltrithylammonium bromide extraction method (CTAB) was used (Mattiucci *et al.,* 2014). DNA obtained was quantified by using the Qubit™ dsDNA HS Assay Kit with Qubit 2.0 (Invitrogen^{TM}) (Sambrook and Russell, 2001).

***Genetic analysis for identification of* A. berlandi**

Because of the possible co-occurrence of *A. pegreffii* from the same definitive host (i.e. *Globicephala melas*) and geographical area (i.e. New Zealand waters), as previously documented (Mattiucci *et al*., 1997), the specimens (N= 46) of *A. berlandi* used in the present study for cross amplification of SSRs loci, were previously identified to the species level by allozyme markers and sequences analysis of the mtDNA *cox2* (Mattiucci *et al.,* 2014). Standard horizontal starch gel electrophoresis was performed at those enzyme loci that have proven to be diagnostic for the species *A. berlandi* (Mattiucci and Nascetti, 2008; Mattiucci *et al.*, 2009). Their staining procedures are those previously reported (Mattiucci *et al.,* 1997). For sequencing the mtDNA *cox2* gene locus, PCR amplification was performed using the primers 211F (5'- TTT TCT AGT TAT ATA GAT TGR TTY AT - 3') and 210R (5' - CAC CAA CTC TTA AAA TTA TC - 3') (Mattiucci *et al.,* 2014; Valentini *et al*., 2006). PCR conditions were those as previously described (Mattiucci *et al.*, 2014).

***Cross - species amplification of microsatellite loci in* A. berlandi**

A set of eight previously identified microsatellite markers (Mattiucci *et al*., 2019), named as *Anisl 00185, Anisl 00314, Anisl 10535, Anisl 07132, Anisl 05784, Anisl 08059, Anisl 00875* and *Anisl 7*, were scored as potentially useful markers on the species *A. berlandi*. Our previously published primer pairs flanking those eight loci (Mattiucci *et al*., 2019) were used to amplify DNA from the 46 selected individual worms. The amplification of microsatellite loci was performed by two Multiplexes PCR: *Anisl 07132, Anisl 05784, Anisl 08059* and *Anisl 00875* by Multiplex 1; while, *Anisl 00185, Anisl 00314, Anisl 10535* and *Anisl 7* by Multiplex 2. Both Multiplex PCR amplifications were 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. The following cycling protocol was used for the amplification both for the two multiplex reactions: 35 cycles with 94°C for 30 sec, 56°C for 90 sec, and 72°C for 60 sec. 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). Individual electropherograms were analysed using the software Genemapper v.4.1 (Applied Biosystems, USA), to determine the genotype of each sample. Patterns of tri- and tetrallelic peaks in the female individuals, as possible results of tissue contamination with sperm from copulation, were not found. Genotyping errors generally associated with microsatellite analysis, such as stutter bands, the presence of null alleles and allelic drop-out were checked by using the software MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al.*, 2004).

Genetic data analysis

The sequences here obtained at the mtDNA *cox-2* gene were aligned by using Clustal X version 2.0 software (Larkin *et al.,* 2007). The number of alleles found at the SSRs loci (A), the observed heterozygosity (H_o), the expected heterozygosity (H_e), the Hardy–Weinberg exact test (Haldane. 1954), the fixation indices (F_{IS}, F_{IT} and the F_{ST}) (Weir and Cockerham, 1984) inferred from the SSRs genetic data sets were evaluated using ARLEQUIN version 3.5 software (Excoffier and Lischer, 2010). Because of the discontinuous range of distribution of the species *A. berlandi* and its genetic sub-structuring in the Pacific Ocean, as previously detected by other nuclear markers (allozymes) (Mattiucci *et al.,* 1997), we have preferred to maintain, for the Analysis of Molecular Variance (AMOVA), the nematode samples, collected from the two individual hosts, as separate sub-populations. AMOVA was used to determine variance among individuals from the two definitive hosts, locus by locus, by using ARLEQUIN version 3.5 (Excoffier and Lischer, 2010) with 1000 permutations. Cavalli-Sforza and Edwards 's chord distance (Cavalli-Sforza and Edwards, 1967) and Nei's distance values (Nei, 1978) were calculated from the SSRs allele frequency estimates, by using BIOSYS 2.0 software program (Swofford and Selander, 1997). An UPGMA (Unweighted Pair Group Method with Arithmetic Mean) was generated by using PHYLIP software (Felsenstein, 1993) based on Nei's (Nei, 1978) distance values.

3.3 RESULTS AND DISCUSSIONS

Identification of* A. berlandi *specimens

Allozyme analysis, of *Anisakis* (*N*= 46 specimens) from the long-finned pilot whale corresponded to *A. berlandi*, according to alleles found at the diagnostic loci with respect to the other members of the *A. simplex* (s. l.) complex, i.e. *Pep C-1*^{92,} and *Mdh-1*^{80,90} (Mattiucci *et al*., 2009). In addition, the sequences of 629 bp in length of the mtDNA *cox2* gene locus

were obtained from the same specimens. According to the diagnostic positions, as previously described (Mattiucci *et al.*, 2014; Valentini *et al.*, 2006), those (N= 46) specimens were assigned to the species *A. berlandi*. The sequences obtained at the mtDNA *cox2* gene (629 bp) of those specimens of *A. berlandi* matched, respectively, the sequences deposited in GenBank for *A. berlandi* from our previous analysis (Mattiucci *et al*., 2014). The new sequences have been submitted to GenBank for the mtDNA *cox2* gene, and their accession numbers are as follows: MN385244, MN385245, MN385246, MN385247.

Genetic diversity within* A. berlandi *based on microsatellite DNA loci

A total of *N*= 24 female and *N*= 22 male adult specimens of *A. berlandi*, i.e., were genotyped at the eight microsatellite loci scored. Each of these markers produced unambiguous genotypes with either a single or double peak on single worms, as anticipated for single locus markers in a diploid organism. Six markers were seen to be polymorphic, with the total number of alleles varying between *A*= 2 (*Anisl 05784*) to *A*= 16 (*Anisl 7*) (Table 1). Whereas, the remaining two loci, i.e. *Anisl 00314* and *Anisl 10535*, were monomorphic in *A. berlandi* specimens here tested (Table 2). For the locus *Anisl 07132*, a certain number of samples repeatedly failed to amplify in *A. berlandi*, suggesting that there were null homozygotes at that locus in the parasite species. The locus exhibited an excess of homozygotes; consistent with this, the observed heterozygotes (H_o) were significantly fewer than the expected heterozygote genotypes (H_e), further suggesting the presence of null alleles at that microsatellite locus (Table 1). Therefore, this marker was not taken into account when other genetic data (i.e. allele frequencies, Cavalli-Sforza (Cavalli-Sforza and Edwars, 1967) and Nei's (Nei, 1978) genetic distance values, as well as F_{ST}) have been considered.

No significant departures from the Hardy-Weinberg Equilibrium (HWE) between observed (H_o) and expected (H_e) heterozygosity were observed at the three polymorphic

scored loci *Anisl 05784, Anisl 08059, Anisl 00875;* while a slight significant value (p= 0.04) was found at the locus *Anisl 00185* (Table 1). Generally, positive values of F_{IS} indicated an excess of homozygote genotypes at the selected loci; whereas, negative values indicate an excess of heterozygote genotypes from the expected HWE (Fig. 1A). Interestingly, *Anisl 7* showed statistically high significant departures from the HWE in *A. berlandi* (Table 1), with a positive F_{IS} value (Fig. 1A). However, when the genotypes at the *Anisl 7* were compared with those observed in adult male and female worms, it was seen that the male worms were homozygous at this locus (F_{IS}= 1) (Fig. 1B). Therefore, the locus *Anisl 7* appeared to be sex-linked in *A. berlandi* because of hemizygosity in the males. In fact, no significant departure (p= 0.13) from the HWE was observed between the observed (H_o= 0.88) and expected heterozygosity (H_e= 0.90), when considering only female worms of *A. berlandi*, scored at the *Anisl 7* locus.

After excluding the SSRs locus (i.e. *Anisl 07132)* affected by null alleles and the sex-linked locus *Anisl 7*, due to hemizygosity of males, the remaining loci showed adequate genetic diversity for population-level genetic analysis. The AMOVA analysis of six nuclear markers showed that a moderate variance was significantly allocated within individuals (≈58%, with F_{IT}= 0.10). Further, a moderate significant (p= 0.03) variation was found among individuals within populations (F_{IS}= 0.11), likely due to the locus *Anisl 00185.*

AMOVA provided no significant genetic differentiation (F_{ST} = -0.01, p =1) between the two populations of *A. berlandi,* here considered, as collected from the two definitive host individuals (Table 2). The last F_{ST} value was at the same degree as those previously observed at the infra-populations levels within the species *A. simplex* (s. s.) and *A. pegreffii*. Indeed, it resulted, on average, F_{ST}≈ 0.008, at the interpopulations level, in *A. simplex* (s. s.) from NE Atlantic waters, and it was, for instance, F_{ST}= 0.002 between pairs of populations

geographically close to each other, such as the two samples of *A. pegreffii* from the Mediterranean Sea (Mattiucci *et al*., 2019).

While, based on the same common SSRs loci scored in the three species of the *A. simplex* (s. l.) complex (excluding the locus *Anisl 00185* found affected by nulls in *A. simplex* (s. s.) and the locus *Anisl 00314* because of its sex-linkage in both *A. simplex* (s. s.) and *A. pegreffii* (Mattiucci *et al*., 2019)), higher and significant levels of F_{ST} were observed, at the interspecific level. Indeed, between *A. berlandi* versus *A. pegreffii* resulted, on average, $F_{ST} \approx$ 0.45, and $F_{ST} \approx 0.57$ *versus A. simplex* (s. s.). Those values are at the same degree as that estimated between *A. pegreffii* and *A. simplex* (s. s.) (on average $F_{ST} \approx 0.33$) (Mattiucci *et al.,* 2019).

Utility of microsatellite markers in* A. berlandi *identification

Allele frequencies calculated at the seven microsatellite loci (excluding *Anisl 07132*, due to the null alleles), are reported in Table 3. Most of the SSR loci studied appeared less polymorphic in the species *A. berlandi* in comparison with the same loci previously investigated (Mattiucci *et al.*, 2019) in the other two species of the complex (Table 3).

On the contrary, in *A. berlandi*, the sex-linked locus *Anisl 7* has shown several alleles; similarly, the locus *Anisl 00185* has shown at least 11 distinct alleles in this *Anisakis* species (Table 3). To do not exclude genetic data set obtained at the sex-linked locus *Anisl 7*, the most reliable estimates of allele frequencies of the parasite species were calculated only in adult specimens, according to the sex-linked genetic model estimate, considering the hemizygosity of the males and the adult female counterpart, as biallelic nematodes at the sex-linked locus (Table 3).

It was also found that most of the amplified loci in *A. berlandi*, seem to share alleles with those previously observed in *A. pegreffii* and *A. simplex* (s. s.) (Mattiucci *et al.,* 2019) (Table 3). However, significant differential allele frequencies in *A. berlandi* with respect to both *A. pegreffii* and *A. simplex* (s. s.) resulted in the scoring of the two SSRs loci *Anisl 00875* and *Anisl 05784* (Table 3, Fig. 2). Indeed, the species *A. berlandi* has shown, for instance, a high frequency (0.98%) of the allele *69* at the locus *Anisl 05784*, while the same allele was scored at very low frequency, i.e. 0.01% and 0.03% in *A. pegreffii* and *A. simplex* (s. s.), respectively (Table 3, Fig. 2). Similarly, at the locus *Anisl 00875 A. berlandi* exhibited a significantly high frequency for the allele *148*, which conversely occurs at very low frequency (0.01%) in both *A. pegreffii* and *A. simplex* (s. s.) (Table 3, Fig. 2). The locus *Anisl 10535* in *A. berlandi* was monomorphic for the allele *128* (Table 3, Fig. 2); while, the same allele was scored at a very low frequency (0.01%) in few populations of *A. pegreffii* and *A. simplex* (s. s.), previously studied at the same locus (Mattiucci *et al.,* 2019) (Table 3, Fig. 2). Therefore, at this locus, the allele observed in *A. berlandi* was seen to be almost diagnostic (at 99%) allowing recognition of *A. berlandi*, with respect to *A. pegreffii* and *A. simplex* (s. s.) (Table 3, Fig. 2). The sex-linked locus *Anisl 7* in *A. berlandi*, showed at least 16 distinct alleles (Table 3, Fig. 2), which are well distinct from two further alleles we observed in the species *A. pegreffii* (Mattiucci *et al*., 2019); thus, the locus was 100% diagnostic between *A. berlandi* and *A. pegreffii*. Finally, the locus *Anisl 00314*, was monomorphic for the allele *100* in the species *A. berlandi* (Table 3); this locus was found to be sex-linked in the species *A. pegreffii* and *A. simplex* (s. s.). Conversely, it was not possible to demonstrate sex-linkage at the locus *Anisl 00314* because its monomorphic status in *A. berlandi*.

Estimates of genetic differentiation by Cavalli-Sforza & Edwards (Cavalli-Sforza and Edwards, 1967) chord distance, *Dc*, inferred from the allele frequencies calculated at those SSRs loci considered as valid (i.e. not affected by null alleles in any of the three *Anisakis*

species) at the interspecific level was, on average, *Dc*= 0.82 and *Dc*= 0.73, between *A. berlandi versus A. pegreffii* and *A. simplex* (s. s.), respectively. For those estimates, allele frequencies data obtained at the loci *Anisl 10535, Anisl 05784, Anisl 08059, Anisl 00875* and *Anisl 7,* were included. Data from *Anisl 00314 and Anisl 00185* were excluded from the estimation because the locus was found affected by nulls in the species *A. simplex* (s. s.), as we have previously demonstrated (Mattiucci *et al.,* 2019). Those values of genetic differentiation were higher than those previously reported from allozyme markers, i.e. on average, D_{Nei}= 0.55 and D_{Nei}= 0.49, between the same pairs comparison (Mattiucci *et al.,* 1997), even if these values were based on larger number of loci. However, those estimates of genetic divergence are at the same scale level as that observed between the two members *A. pegreffii* and *A. simplex* (s. s.), when based on the same SSRs markers; indeed, *Dc*= 0.62 between *A. pegreffii* and *A. simplex* (s. s.) (Mattiucci *et al.*, 2019).

Conclusions

In the present study, the utility of cross-species transfer of microsatellites, previously developed in the other two closely related species of the *A. simplex* (s. l.) complex, was validated for the genotyping of *A. berlandi*. Out of the eight SSRs previously scored, only one, i.e. the locus *Anisl 07132,* failed in the cross-amplification in this *Anisakis* species. Null alleles have been previously detected in other SSRs loci in the species *A. simplex* (s. s.) (i.e. *Anisl 00314* and *Anisl 00185*); this is because the SSRs primers were first selected in the species *A. pegreffii* (Mattiucci *et al.*, 2019). The possible presence of null alleles would require careful protocol development in order to obtain consistent amplification, when cross-species amplification is tested between closely related species.

An interesting discovery in this study was that in *A. berlandi* the SSR locus *Anisl 7* was located on the X sex chromosome, thus being sex-linked. Indeed, as in the case of *A.*

pegreffii and *A. simplex* (s. s.), males of *A. berlandi* are hemizygous at that locus for several alleles. This finding gives further support to the generalisation that male specimens belonging to *Anisakis* spp. are likely to possess the XO sexual karyotype, as other ascarids show (Müller and Tobler, 2000).

In spite of the low number of SSRs loci so far developed in the three species of the *A. simplex* (s. l.) complex and the finding of some of them not properly cross-amplifying in all the three species, the actual SSRs loci to be considered as "valid" nuclear markers, are of potential value in the discrimination of the three species (Table 3, Fig. 2). Indeed, for instance, the locus *Anisl 10535* which has shown the same alleles without significant difference in their relative proportions in *A. pegreffii* and *A. simplex* (s. s.), was found to be fixed, instead, with a single allele in *A. berlandi*; while the same allele very rarely occurred (0.01%) in the other two species (Table 3, Fig. 2). Similarly, the locus *Anisl 7*, which was diagnostic, at 100%, between *A. pegreffii* and *A. simplex* (s. s.) (Mattiucci *et al.*, 2019), also had full diagnostic value between *A. pegreffii* and *A. berlandi*. In the present genetic analysis, no individuals showing evidence of mixed ancestry genotypes were detected between *A. berlandi* and *A. pegreffii*, despite the collection of *A. berlandi* specimens from a geographical area where the sympatry between *A. pegreffii* and *A. berlandi* could occur (Mattiucci *et al.*, 2018a).

SSRs nuclear markers here studied also showed the clear distinction of *A. berlandi*, from the other two taxa of the same complex, as inferred from the F_{ST} and *Dc* genetic differentiation values (Fig. 3). Interestingly, the topology of the clustering analysis (Fig. 3), here obtained by UPGMA, appears similar to that observed and inferred, in our previous SSRs studies (Mattiucci *et al*., 2019), as well as from mitochondrial and nuclear markers (Mattiucci *et al.*, 2014; Valentini *et al*., 2006). The clear distinctiveness of the taxon *A.*

berlandi, as stated above, also highlights the utility of the detected SSRs in the identification of this species, since SSRs achieve a high-discriminatory power in a nuclear multilocus genotyping approach.

The validated SSRs loci in the present and our previous studies (Mattiucci *et al.*, 2019), will be applicable for future investigation of population genetic structure, at the intraspecific level, in *A. berlandi* collected from intermediate/paratenic and definitive hosts of other oceanographic waters, where the species also occur. Because of the discontinuous range of *A. berlandi* including Pacific Canada and Austral Regions (Mattiucci *et al*., 2018a), possible data acquired in future analysis, based on SSRs scoring, and gene flow estimation, would add knowledge about the genetic sub-structuring of this parasite species, as we previously detected by allozyme markers (Mattiucci *et al*., 1997). In that aim, allozyme analysis, despite its powerful role in population genetics analysis and species detection of anisakids, has the disadvantage to be not used as a standardized method. Whereas, SSRs have demonstrated to be suitable and standardized nuclear tool to investigate genetic variability and population genetic structure of the other two members of the *A. simplex* (s. l.) complex, i.e. *A. pegreffii* and *A. simplex* (s. s.). (Mattiucci *et al.*, 2019).

In addition, scoring of the SSRs loci in other larval and adult populations of *A. berlandi*, would clarify if the F_{IT} value higher than zero here observed in *A. berlandi*, as we have previously found in both *A. pegreffii* and *A. simplex* (s. s.) (Mattiucci *et al*., 2019), indicates that a certain subdivision between subpopulations of the parasite species, hosted by different definitive and intermediate/paratenic fish hosts, would exist. However, the high polymorphism observed at the scored SSRs loci, requires a larger number of specimens to be studied in the parasite populations collected from different definitive and

intermediate/paratenic host species, in order to find causes of those differences at the infrapopulation level.

Finally, SSRs markers provide a powerful means to investigate, in a multi-nuclear genotyping approach, including also SNP polymorphisms detected in other nuclear genes (Mattiucci *et al*., 2016; Palomba *et al.*, 2019), the detection of possible patterns of hybridization/introgression events between the three species of the *A. simplex* (s. l.) complex, in sympatric areas and syntopic conditions, where they do occur (Mattiucci *et al.*, 2018a).

3.4 TABLES AND FIGURES

Table 1. Genetic diversity at six microsatellite loci of adult specimens of *A. berlandi*, analysed in the present study. Loci *Anisl 00314* and *Anisl 10535* were monomorphic. *N*= total number of genotyped nematodes at each locus; H_e= expected heterozygosity; H_o= observed heterozygosity; *A*= number of alleles detected at each locus; *p*= indicates the significance *(p*<0.05) value of the deviation from HWE expectation.***p<<0.001

Locus		
Anisl 00185	N	46
	H_o	0.65
	H_e	0.85
	p-value	0.04
	A	11
Anisl 07132	N	12
	H_o	0.25
	H_e	0.81
	p-value	***
	A	6
Anisl 05784	N	46
	H_o	0.04
	H_e	0.04
	p-value	1.00
	A	2
Anisl 08059	N	46
	H_o	0.37
	H_e	0.31
	p-value	0.43
	A	3
Anisl 00875	N	46
	H_o	0.09
	H_e	0.09
	p-value	1.00
	A	3
Anisl 7	N	46
	H_o	0.43
	H_e	0.89
	p-value	***
	A	16

Table 2. AMOVA results for *A. berlandi* collected from different individual hosts. d.f.= degrees of freedom; *$p<0.05$; n..s.= not significant.

Source of variation	d.f.	Sum of squares	Variance components	% of variation	*F*-statistics
among populations	2	0.937	-0.010	-1.61	F_{ST} -0.016 (n.s.)
among individuals within populations	43	31.096	0.073	11.50	F_{IS}= 0.113 *
within individuals	46	26.500	0.576	90.11	F_{IT}= 0.100 *
total	91	58.533	0.639		

Table 3. Allele frequencies observed at seven microsatellite loci tested in *A berlandi*, in comparison with those we previously reported in *A. pegreffii* and *A. simplex* (s.s) at the same SSRS loci (Mattiucci et al., 2019). With regard to the polymorphic sex-linked locus *Anisl 7*, 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; *(ii)* their adult female counterparts, as biallelic at the sex-linked loci. The frequencies of *Anisl 00185* and *Anisl 00314* are not reported in *A. simplex* (s. s.), because those loci were affected by null alleles, as we previously found (Mattiucci *et al*., 2019).

		Anisakis berlandi	*A. pegreffii*	*A. simplex* (s. s.)
Locus				
Anisl 00185	Allele			
	182	0.01	-	-
	185	0.01	0.01	-
	188	0.01	0.04	-
	191	0.11	0.07	-
	194	0.15	0.25	-
	197	0.18	0.24	-
	200	0.28	0.15	-
	203	0.09	0.18	-
	206	0.07	0.04	-
	209	0.07	0.01	-
	212	0.02	0.01	-
Anisl 00314	Allele			
	96	-	0.05	-
	100	1.00	0.32	-
	104	-	0.22	-
	108	-	0.25	-
	112	-	0.13	-
	116	-	0.01	-
	120	-	0.01	-
	124	-	0.01	-
Anisl 10535	Allele			
	125	-	0.01	0.01
	128	1.00	0.01	0.01
	131	-	0.02	0.02
	134	-	0.10	0.91
	137	-	0.14	0.04
	140	-	0.51	0.01
	143	-	0.18	-
	146	-	0.02	-
	149	-	0.01	-
Anisl 05784	Allele			
	57	-	-	0.01
	60	-	-	0.01
	63	-	0.01	0.21
	66	-	0.01	0.04
	69	0.98	0.01	0.03
	72	0.02	0.01	0.01
	75	-	0.01	0.02
	78	-	0.02	0.46
	81	-	0.05	0.13
	84	-	0.07	0.05
	87	-	0.22	0.01
	90	-	0.31	0.01
	93	-	0.18	-
	96	-	0.05	0.01
	99	-	0.03	-
	102	-	0.01	-
	105	-	0.01	-

(continues)

(continued)

		Anisakis berlandi	*A. pegreffii*	*A. simplex* (s. s.)
Locus				
Anisl 08059	Allele			
	78	-	-	0.01
	82	-	0.01	0.03
	86	0.80	0.24	0.85
	90	0.19	0.03	0.05
	94	0.01	0.12	0.01
	98	-	0.13	0.02
	102	-	0.19	0.01
	106	-	0.12	0.01
	110	-	0.06	0.01
	114	-	0.04	-
	118	-	0.02	-
	122	-	0.01	-
	126	-	0.01	-
	130	-	0.01	-
	134	-	0.01	-
Anisl 00875	Allele			
	142	-	-	0.01
	145	-	0.01	0.01
	148	0.95	0.01	0.01
	151	0.04	0.05	0.01
	154	0.01	0.03	0.01
	157	-	0.67	0.20
	160	-	0.16	0.53
	163	-	0.04	0.16
	166	-	0.01	0.04
	169	-	-	0.01
	172	-	0.01	0.01
	175	-	0.01	-
Anisl 7	Allele			
	216	-	0.01	-
	219	-	0.22	-
	222	-	0.75	-
	225	0.02	0.02	-
	228	0.02	-	-
	243	0.02	-	-
	252	0.02	-	0.13
	255	-	-	0.48
	258	0.02	-	0.18
	261	0.07	-	0.11
	264	0.02	-	0.04
	267	0.20	-	0.03
	270	0.17	-	0.02
	273	0.11	-	-
	276	0.07	-	-
	279	0.09	-	-
	282	0.11	-	-
	285	0.02	-	0.01
	288	0.02	-	-
	294	0.02	-	-

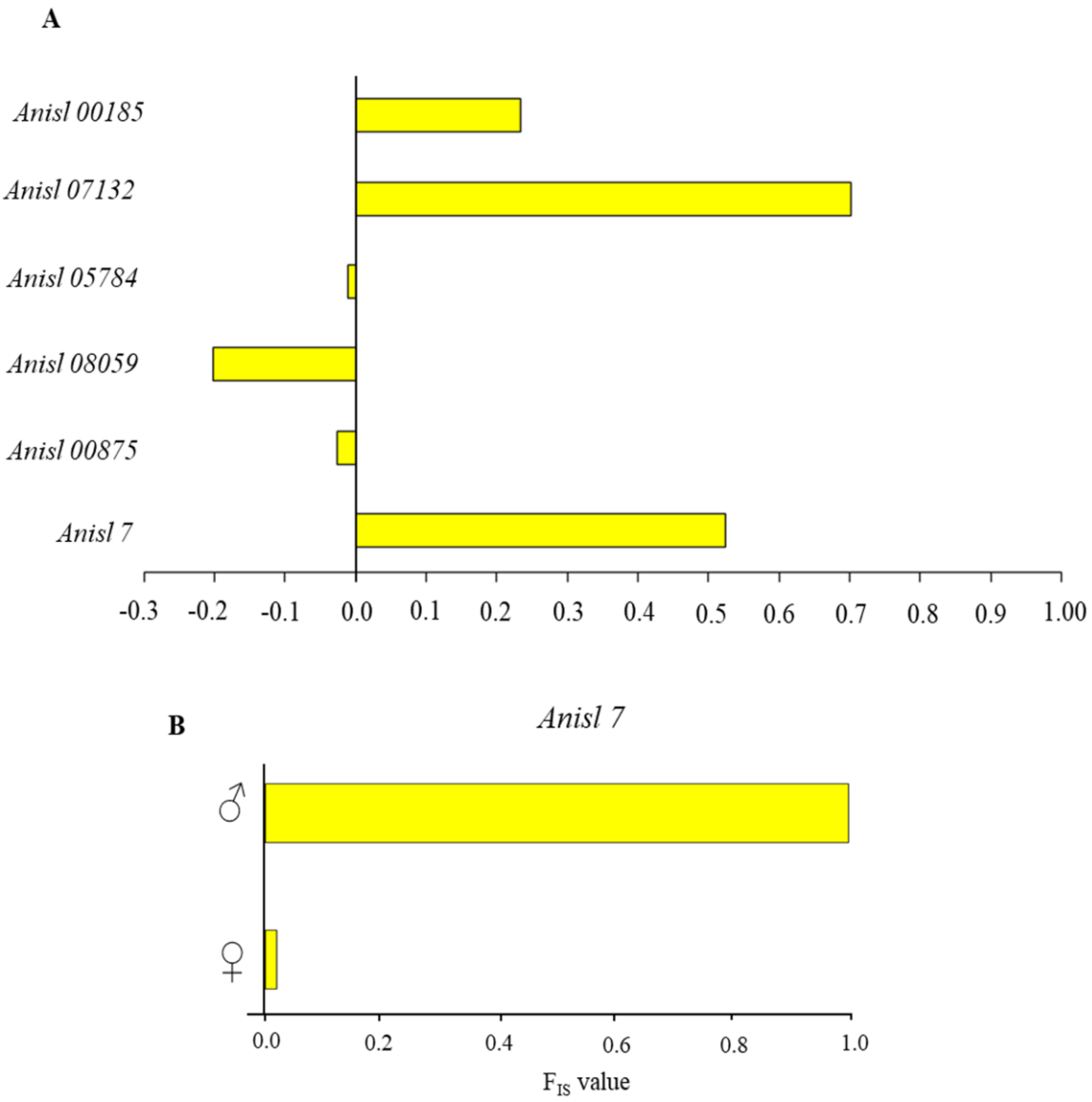

Figure 1: (A) F_{IS} calculated at the six microsatellite loci among the eight studied in *A. berlandi*. Two loci (i.e. *Anisl 00314* and *Anisl 10535*) were not included because they were found to be monomorphic. Negative values indicate heterozygous excess, while positive values indicate homozygous excess from that expected under Hardy–Weinberg Equilibrium (HWE); (B) F_{IS} in male and female specimens of *A. berlandi* at the sex-linked locus *Anisl 7*.

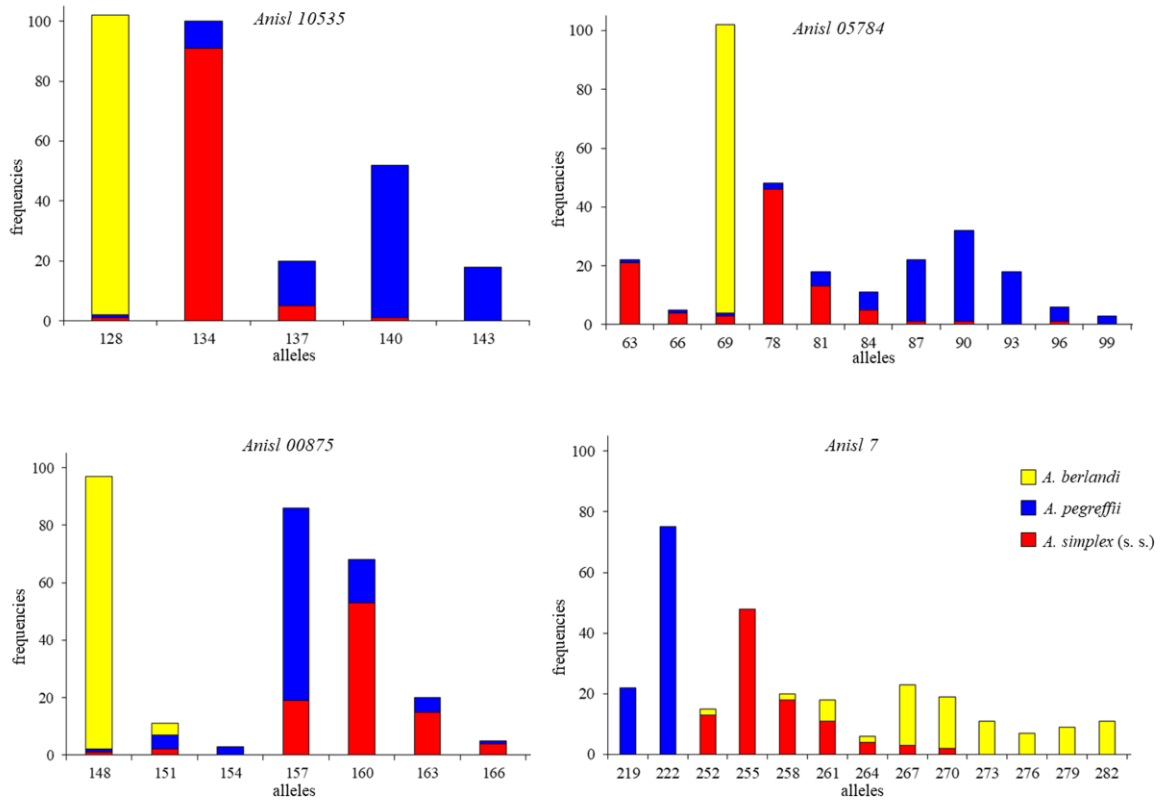

Figure 2: Distribution of allele frequencies of microsatellite loci in *A. berlandi* (yellow color) shown for those partially diagnostic loci, with respect to the species *A. pegreffii* (blue color) and *A. simplex* (s. s.) (red color) (see Table 3). Alleles showing a frequency ≤0.03 in the three *Anisakis* spp. were not included in the graphical representation, except in those case when they occurred in common with one or in the other two *Anisakis* species at a frequency ≥0.03 (see Table 3).

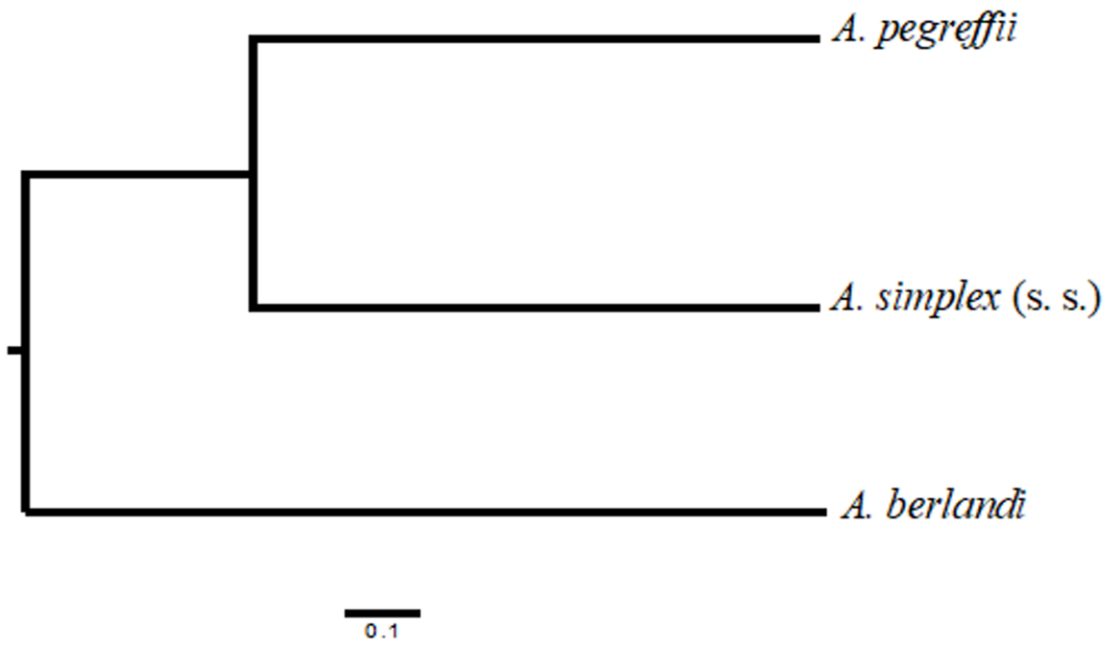

Figure 3: The unweighted pair group method of analysis (UPGMA) cluster based on Nei's genetic distance values, inferred from allelic frequencies calculated at five microsatellite loci (i.e. *Anisl 10535*, *Anisl 05784*, *Anisl 00875*, *Anisl 08059*, *Anisl 7*), showing the genetic relationship between *A. berlandi* and the other two members of the *A. simplex* (s. l.) (based on the allele frequencies found, on average, at the same loci in *A. pegreffii* and *A. simplex* (s. s.) in our previous analysis (Mattiucci *et al*., 2019).

4. STUDY 3

MULTILOCUS GENOTYPING APPROACH FOR THE RECOGNITION OF *ANISAKIS PEGREFFII* AND *A. BERLANDI* (NEMATODA: ANISAKIDAE), WITH THE FIRST EVIDENCE OF HYBRIDIZATION BETWEEN THEM IN THE SYMPATRIC AREA FROM OFF THE NEW ZEALAND COAST.

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(In preparation)

Abstract

Basin water from off the New Zealand coast is reported as one of the sympatric areas of *Anisakis pegreffii* and *A. berlandi*. In the present study, a total of N= 316 nematodes collected from three cetaceans species and eight fish species were identified as belonging to the species *A. pegreffii* (*N*= 180), or to *A. berlandi* (*N*=133), as inferred from a multilocus genotyping approach. In particular, a panel of seven SSRs loci previously developed (Mattiucci *et al.,* 2019; Bello *et al.,* 2020) in the species of the *A. simplex* (s.l.) complex, and four scored by Mladineo *et al.* (2017), *plus* the sequences at a novel diagnostic nuclear gene *nas10* nDNA, recently validated in the species of *A. simplex* (s.l.) (Palomba *et al.*, submitted) complex, were applied. Out of those four SSRs newly scored in the present study in the two considered species, the loci *Anisl* 15 and *Anisl* 2 have shown fixed alternative alleles between *A. pegreffii* and *A. berlandi*; i.e. they resulted to be 100% diagnostic between the two species. Whereas, the locus *Anisl* 22 resulted to be partial diagnostic between them. Out of the sex-linked loci

previously detected in the two parasite species, further two loci, i.e. *Anisl* 4 and *Anisl* 22, were demonstrated to be sex-linked in the species *A. pegreffii*; *Anisl 4* resulted to be sex-linked in *A. berlandi*, while *Anisl 22* resulted to be monomorphic, thus it was not possible to demonstrate the sex-linkage. The newly scored SSRs loci were validated on a large numbers of individuals of the two species. According to the Bayesian clustering algorithms, based on those SSRs diagnostic and partially diagnostic SSRs *plus nas10* nDNA, markers, one adult and two L3 individuals were recognized as F1 hybrids between the two taxa. This represents the first detection of current hybridization between the two related species, in a sympatric area. Finally, because of the sex-linkage of some of the scored SSRs loci, it was possible the sex determination of the larval stages of the two species.

Keywords*: Anisakis pegreffii*, *Anisakis berlandi*, SSRs loci, Sex-linked loci, *nas10*, Hybridization.

4.1 INTRODUCTION

The Pacific basin water from off the New Zealand coast has been reported as a sympatric area of *A. pegreffii* and *A. berlandi* (Mattiucci *et al*., 1997), sibling species of the *A. simplex* (s. l.) complex (Mattiucci *et al*., 2014). In the Austral regions, the two parasite species have been found in South-West Atlantic (off Argentine coast), South-East Atlantic waters (off African coast), as well as from off Chilean coast (for a review, Mattiucci *et al*., 2018). In the Pacific waters of the Austral Region, the two species have been identified as adult parasites co-infecting the same definitive hosts *Globicephala melas* and *Grampus gryseus* (Mattiucci *et al*., 2018). Fourth-stage larvae of *A. berlandi* were identified also in *Kogia sima* from Australian waters (Shamsi *et al.*, 2012) and as L3 in other unusual hosts (Shamsi *et al.*, 2017).

Despite their sympatrically occurrence, even in syntopy in the same definitive hosts, the occurrence of hybrid categories between *A. pegreffii* and *A. berlandi* were not so far recorded. It has been frequently reported that sympatric populations of closely related parasitic nematodes might interbreed (Anderson, 2001; Criscione *et al*. 2007; Dunams-Morel *et al.* 2012), including species of the genus *Anisakis* (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; Mattiucci *et al.*, 2016). However, hybridization, introgression and the retention of ancestral polymorphisms in closely related anisakid nematodes could generate patterns of genetic variation which complicate their disclosure, when choosing inappropriate genetic/molecular markers and/or when the detection of hybrids categories is inferred by genetic markers obtained from the analysis of a single locus (Mattiucci *et* al., 2016; 2018).

The recognition of those parasite species has been so far obtained by the direct sequences analysis of the mtDNA *cox2,* allowing to distinguishing the *A. pegreffii* and *A. berlandi* from the other species of the genus *Anisakis,* which could be also sympatrically

found in the same geographical areas, such as the New Zealand waters (for a review Mattiucci *et al*., 2018). Among the diagnostic nuclear markers so far available to recognize the two parental species *A. pegreffii* and *A. simplex* (s. s.) and their possible mixed ancestry, there are the allozymes (Mattiucci *et al.,* 1997), the direct sequences analysis and the RFLPs-PCR of the ITS region of rDNA (D'Amelio *et al*., 2000). However, concerning these nuclear markers, in the case of allozymes, biparentally inherited codominant markers, despite of their utility in demonstration of reproductive isolation are not a standardised approach, available in all the laboratories; further they are requiring fresh or frozen specimens which is not always possible to be obtained. As regards to the markers at the ITS region of rDNA, the genotypes inferred from PCR–RFLPs analysis of ITS rDNA cannot be included in the analysis of STRUCTURE, because the ITS region of rDNA undergoes concerted evolution (Elder and Turner, 1995; Ganley and Kobayashi, 2007), a phenomenon which violates the assumption of Hardy–Weinberg, necessary to perform elaboration by clustering genotyping STRUCTURE analysis. On the other hand, a nuclear multilocus genotyping approach to be used as a powerful mean to investigate, various microevolutionary aspects in anisakid taxa, as well as the detection of possible patterns of hybridization/introgression events between *Anisakis* spp. in sympatric areas (Mattiucci *et al.,* 2016; 2019).

In this regards, in the last recent years, the looking for further nuclear markers to be useful to recognize the parental species of the *A. simplex* (s. l.) complex (i.e. *A. simplex* (s. s.), *A. pegreffii* and *A. berlandi*), and their mixed ancestry in their overlapping range of distribution, has been attempted (Mattiucci *et al.,* 2016; Mladineo *et al*., 2017; Mattiucci *et al.,* 2019; Bello *et al.,* 2020; Palomba *et al*., submitted). Among those investigations, a set of 8 novel markers developed from DNA microsatellite loci, revealed to be useful in their diagnostic power, to recognize species *A. pegreffii* and *A. simplex* (s.s.) (Mattiucci *et al.,* 2019), as well as *A. berlandi* (Bello *et al*., 2020): Additionally, in the same aim to discovery

new markers, diagnostic nucleotide positions between *A. pegreffii* and *A. simplex* (s.s.) at the EF 1alpha nDNA gene locus, were found (Mattiucci *et al*., 2016). Similarly, further diagnostic SNPs, allowing the correct assignment of individuals to the three species *A. pegreffii, A. simplex* (s.s.) and *A. berlandi*, were discovered the gene locus of *nas10* nDNA (Palomba *et al.*, submitted).

In the present paper, a Bayesian clustering of genotypes obtained from multi-marker nuclear loci analysis of individual nematodes of *Anisakis* spp. collected from a sympatric area of the two species *A. pegreffii* and *A. berlandi* was carried out in order to: *(i)* correctly identify specimens belonging to the parental taxa *A. pegreffii* and *A. berlandi*; *(ii)* compare the assignment of each *Anisakis* specimen obtained by eleven SSRs loci [i.e. 8 developed in Mattiucci *et al.*, (2019) and four in Mladineo *et al*., (2017)], *plus* the sequence analysis of a novel nuclear DNA locus (i.e. *nas 10* nDNA); *(iii)* recognize possible hybrid categories between the two species*; (iv)* to attempt the sex-determination of larval specimens (L3 and L4 stage) of the two parasite species, as inferred from sex-linked SSRs loci.

4.2 MATERIALS AND METHODS

Sampling

A total of N= 316 nematodes belonging to *Anisakis* spp. were examined in the present study. The only sampling area here considered included the waters along the New Zealand coast (44°30’ S–172°58’ E): those waters are included in the geographical range of the two species *A. pegreffii* and *A. berlandi*, from where they are reported as sympatric (Mattiucci *et al.* 2018a). Specimens of *Anisakis* spp. were collected from eight fish species as larval stages (L3), and from three cetacean species as L4 and adult stages. Details concerning the life-history stage, intermediate/paratenic (fish) and definitive hosts (cetaceans) of the *Anisakis* spp. specimens examined in this study were given in Table 1.

Anisakis spp. adults were collected during the years 2011-2012 from cetaceans stranded on the coast of New Zealand, i.e. three pilot whales, *Globicephala melas* (Traill), one Risso's dolphin, *Grampus griseus* (Cuvier) and one Hector's dolphin, *Cephalorhynchus hectori* (van Beneden), while the other samples at larval stage were collected during a sampling in New Zealand, by fish species caught during the winter in 2018 (Table 1). The nematodes were repeatedly washed in saline solution and then preserved in 70% alcohol. Considering the sex-linkage of microsatellite loci, as obtained in the previous studies (Mattiucci *et al.*, 2019; Bello *et al*., 2020), the adult specimens were first distinguished as females and males. Sex discrimination was performed according to the main morphological diagnostic features between sexes (Mattiucci *et al*., 2018a), by the use of an optical microscope, at a total magnification of X100–400. Number of adult female and male specimens was reported in Table 1. A little part of the worm's body, as much as possible far from the female uterus, to avoid sperm male contamination, was used for the molecular analysis.

Laboratory procedures

DNA extraction

The total DNA was extracted by using the cetyltrithylammonium bromide method (CTAB), as detailed elsewhere (Mattiucci *et al*. 2014). For the DNA extraction, *Anisakis* at L3 larval stage were used *in toto,* while a tissue portion of 2mg was used from each adult and L4 larval specimen (Table 1).

Genetic identification of* Anisakis *spp. specimens

All the specimens (*N*= 316) were previously identified, at the species level by using sequences analysis at the mtDNA *cox2* (Valentini *et al*., 2006; Mattiucci *et al*., 2014) and *metallopeptidase 10 nas10* nDNA (Palomba *et al., sub.*) gene loci.

For the direct sequencing of the mtDNA *cox2* gene, PCR amplification was performed using the primers 211F (5'- TTT TCT AGT TAT ATA GAT TGR TTY AT - 3') and 210R (5' - CAC CAA CTC TTA AAA TTA TC - 3') (Nadler and Hudspeth, 2000; Mattiucci *et al*., 2014). Thermal PCR protocol was performed using the following conditions: 94°C for 3 min (initial denaturation), followed by 34 cycles at 95°C for 30 sec (denaturation), 46°C for 60 sec (annealing) and 72°C for 90 sec (extension), and a final post-amplifications step at 72°C for 10 min.

PCR amplification was carried out in an volume of 25 µL volume containing 0.5 µL of each primer 10 mM, 2.5 µL of $MgCl_2$ 25 mM (Promega), 1.5 µL of PCR buffer 5x (Promega), DMSO 0.08 mM, 0.5 µL of dNTPs 10 mm (Promega), 5 U of HotStart Go-Taq Polymerase (Promega) and 2 µL of total DNA (Mattiucci *et al*., 2014).

For the direct sequencing of the *nas10* gene PCR amplification was performed 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′) (Palomba *et al*., submitted). The PCR conditions were those reported in details in Palomba *et al*. (sub.).

PCR amplification of SSR markers and genotyping

All the *A. pegreffii* and *A. berlandi* specimens previously identified by mtDNA *cox-2* and *nas10* genes were analyzed at the panel of six microsatellite loci among those developed in Mattiucci *et al*. 2019, (i.e. *Anisl 00185, Anisl 00314, Anisl 10535, Anisl 05784, Anisl 08059*

Anisl 00875) previously scored in the species *A. pegreffii* (Mattiucci *et al*., 2019) and in *A. berlandi* (Bello *et al*., 2020). Additionally, the locus *Anisl 7* (Mladineo *et al*., 2017), already scored in both *A. pegreffii* (Mattiucci *et al*., 2019) and *A. berlandi* (Bello *et al.*, 2020) was furtherly used in the present study. Finally four microsatellite loci (i.e., *Anisl 4, Anisl 22, Anisl 15* and *Anisl 2),* among those developed in Mladineo *et al*., (2017a) were tested for the first time in our specimens Thus, a total of eleven SSRs loci were tested in the N= 316 specimens analyzed in the present study.

The amplification of microsatellite loci was performed in three Multiplex PCR: *Anisl 05784, Anisl 08059* and *Anisl 00875* in Multiplex 1; *Anisl 00185, Anisl 00314, Anisl 10535* and *Anisl 7* in Multiplex 2; *Anisl 4, Anisl 22, Anisl 15 and Anisl 2* in Multiplex 3. All the Multiplex PCR amplifications were 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 both for the two multiplex reactions: 35 cycles with 94°C for 30 sec, 56°C for 90 sec and 72°C for 60 sec. 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 analyze 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).

Genetic data analysis

As previously reported, for distinguishing *A. pegreffii* specimens from *A. berlandi* ones, the sequences obtained at the mtDNA *cox-2* (Valentini *et al.*, 2006; Mattiucci *et al*., 2014) and *nas10* (Palomba *et al*., submitted) genes were aligned by using Clustal X version 2.0 software (Larkin *et al*., 2007).

ARLEQUIN version 3.5 software (Excoffier and Lischer, 2010) was used to evaluate the number of alleles per locus (A), the observed heterozygosity (H_o), the expected heterozygosity (H_e), the Hardy–Weinberg exact test (Haldane, 1954; Weir, 1996) and the fixation index F_{IS}. Significance levels were adjusted using the sequential Bonferroni correction for multiple tests (Rice, 1989).

For evaluating population genetic structure considering those nuclear markers (microsatellites and *nas10*) and for evaluating gene exchange between species, STRUCTURE 2·3·3 software (Pritchard *et al.*, 2000) was used. STRUCTURE is a model-based procedure that uses individual multi-locus genotypes to identify the optimal number of clusters (K) in a dataset, by minimizing the resulting Hardy-Weinberg and linkage disequilibria. The analysis by STRUCTURE was performed in a multilocus genotyping approach and twenty replicates of the analysis were carried out to check for consistency; each run for 100000 MCMC iterations, following a burn-in of 50000 iterations, under the admixture model and the assumption of correlated allele frequencies between the two species studied. 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 optimality criteria (Evanno *et al.*, 2005).

4.3 RESULTS

Identification of Anisakis *spp. specimens by sequences analysis of the mtDNA* cox2 *and* nas10 *nDNA gene loci*

The alignment of the obtained sequences at the mtDNA *cox-2* gene locus (629 bp) allowed to assign *N*= 180 specimens to *A. pegreffii* species, while *N*= 136 were assigned to *A. berlandi* according to the diagnostic positions between *A. pegreffii* and *A. berlandi* species, as reported in Valentini *et al*. (2006) and Mattiucci *et al*. (2014), (Table 2). While, the assignment of N= 180 nematodes to the species *A. pegreffii*, and N= 133 as *A. berlandi* (Table 2) was achieved by the sequences analysis at the nuclear gene locus *nas 10* nDNA considering the diagnostic positions between the two taxa (Palomba *et al*., submitted). In particular, according to the diagnostic positions detected at the last nuclear gene locus, between the three species of the *A. simplex* (s. l.), the specimens of both *A. pegreffii* and *A. berlandi* showed always the "C" allele at the position 173 (which is diagnostic with respect to the species *A. simplex* (s.s.)); whereas, at the diagnostic position 251, all (N= 180) individuals of *A. pegreffii* showed the "G" allele, while the *A. berlandi* specimens (N= 131) have shown always a "C" allele (which is the diagnostic position between *A. pegreffii* and *A. berlandi*). Finally, three specimens, among those identified as *A. berlandi* (N= 3) at the mtDNA *cox-2* gene locus, showed a heterozygous genotype "C/G" at the diagnostic position 251 of the *nas10* gene locus; indeed they showed overlapping peaks (Figure 1).

Genetic diversity of SSRs in A. pegreffii *and* A. berlandi

The total 316 nematodes previously identified at the *nas 10* nDNA, were genotyped at eleven microsatellite loci, (Table 2). Any specimens of the dataset showed pattern consisted of three peaks; thus, it could exclude contamination with sperm's male in the female worms.

In *A. pegreffii*, as also reported in Mattiucci *et al*. (2019), the loci *Anisl 00185*, *Anisl 00314*, *Anisl 10535*, *Anisl 05784*, *Anisl 08059*, *Anisl 00875* and *Anisl 7* were polymorphic; in the present study, the mean number of alleles varied from A= 4 at the locus *Anisl 7*, while it resulted to be A= 14 at locus *Anisl 08059* (Table 3). The new SSRs loci scored in the present study, in *A. pegreffii*, i.e. *Anisl 4* and *Anisl 22*, showed a high level of polymorphism in this taxon, showing a maximum number of 14 and 7 alleles, respectively (Table 3).

As regards to *A. berlandi*, as also reported in Bello et al., (2020), the loci (i.e. *Anisl 00185*, *Anisl 05784*, *Anisl 08059*, *Anisl 00875*, *Anisl 7*) were polymorphic. In Bello *et al.*, (*in press*) the locus *Anisl 10535* resulted to be monomorphic; while, in this work it showed another allele in addition to the most common one, i.e. the allele *131* (Suppl. Table 1). At the newly tested SSRs loci, *A. berlandi* showed a high number of alleles (16) at the locus *Anisl 4*, while, it was monomorphic at the *Anisl 22*. (Table 3; Suppl. Table 1).

Finally, the two loci, newly tested, i.e. *Anisl 15* and *Anisl 2*, resulted to be completely monomorphic in the both species. Thus, *A. berlandi* showed four monomorphic microsatellite loci (i.e. *Anisl 00314, Anisl 22, Anisl 15* and *Anisl 2*).

Deviations from the Hardy-Weinberg Equilibrium (HWE) were tested at each locus for the two species (Table 3, Fig. 2a). No high statistically significant departure from the HWE resulted at the loci *Anisl 00185*, *Anisl 10535*, *Anisl 05784*, *Anisl 08059* and *Anisl 00875*, in both *A. pegreffii* and *A. berlandi* populations (Table 3, Fig. 2a).

In Mattiucci *et al*. (2019) and Bello *et al*. (*in press*), the loci *Anisl 00314* and *Anisl 7* had already shown departures from the equilibrium and they were proved to be sex-linked, both in *A. pegreffii* and *A. berlandi* species. Thus, in the present study too, considering the adult female specimens only to perform the HWE analysis, the p-value resulted to be not significant in both loci and species (Table 4, Fig. 2b).

About the SSR loci newly scored in the present study, i.e. *Anisl 4* and *Anisl 22*, after excluding the occurrence of stutter bands, null alleles and allele drop-out, was supposed for those loci a sex-linkage too. In order to confirm that hypothesis, the genotypes analyzed at *Anisl 4* and *Anisl 22* loci in the adult samples of *A. pegreffii*, and the genotypes analyzed at *Anisl 4* locus only in adult samples of *A. berlandi*, were split based on the sex of worms. Thus, it was found that all male specimens were homozygous at those considered loci (also for different alleles) (F_{IS} =1) (Fig. 2b), while the female specimens did not deviate from the HWE (Table 4, Fig. 2b). Sex-linkage was confirmed in *Anisl 4* and *Anisl 22* loci for the species *A. pegreffii*. ; In the case of *A. berlandi* only *Anisl 4* resulted to be sex-linked; as regards *Anisl 22* in this taxon, the monomorphic status of this locus did not allow to evaluate the sex-linkage.

X-linked loci and assignment of sex in larval stages of* A. pegreffii *and* A. berlandi *species

In the present study, four sex-linked loci were available for sex-determination for *A. pegreffii* species (i.e. *Anisl 00314, Anisl 7, Anisl 4* and *Anisl 22*), and two for *A. berlandi* species (i.e. *Anisl 7* and *Anisl 4*), because of the monomorphism in the two others.

Because of the sex-linkage, the larval stage specimens of the present dataset, which showed a heterozygote genotype at least at one of those sex-linked loci, this condition was considered as female. According to this assumption,,as regards *A. pegreffii*, 55 L3 and L4 showing heterozygote genotypes at those 4 sex-linked loci, were assigned to female gender and added to the 58 adult females. Whereas, 52 L3and L4 larval stages of *A. berlandi*, being heterozygotes at the two loci, resulted to be females and added to the 22 adult females. Thus, the H-W equilibrium was again calculated at sex-linked loci on a total of assumed females of the two species: i.e. N= 113 of *A. pegreffii* and N= 74 of *A. berlandi*. No statistical deviation from HWE was found.(Table 5).

However, the statistical occurrence of homozygous specimens at those X-linked loci (four in *A. pegreffii* and two in *A. berlandi* species) was also calculated. Starting from the expected heterozygosis at the four sex-linked loci in *A. pegreffii* female specimens only (see Table 5), we have calculated the expected homozygosis at the same loci. Therefore, in *A. pegreffii* females the expected homozygosis resulted to be 0.25 for *Anisl 00314*, 0.67 for *Anisl 7*, 0.18 for *Anisl 4* and 0.41 for *Anisl 22* (see Table 5). The product of those values presented the expected probability of homozygous females at all four X-linked loci here considered. So, in this case, 1,2% of larvae are expected females homozygous at those loci. Considering thedataset, since the larval homozygous specimens at all four sex-linked loci were 31, it was supposed that they were all males, and thus not homozygous but hemizygous (Table 6).

As regards to the species *A. berlandi* , the sex determination was based on only two sex-linked loci (i.e. *Anisl 7* and *Anisl 4*), because the two other X-linked resulted to be monomorphic (i.e. *Anisl 00314* and *Anisl 22*). Although for this taxon only two sex-linked loci were available to discriminate sex in larval stages, the results obtained from statistical calculations could be considered reliable. In fact, *Anisl 7* and *Anisl 4* loci showed (i) a very high level of genetic variability, since their number of alleles (A) was 28 and 16, respectively, (ii) and their observed heterozygosis (H_o) resulted to be 1.00 and 0.77, respectively. Therefore, the expected probability to find female specimens showing homozygous genotypes at both the considered sex-linked loci would be very low. In fact, the expected homozygosis at the two sex-linked loci resulted to be 0.08 for *Anisl 7* and 0.17 for *Anisl 4* (see Table 5); thuis, the product of those values resulted to be around the 1%. Thus, the percentage of homozygous females at both those loci was 1% in *A. berlandi* species. Here too, the N of homozygous worms at the considered loci was 42, and so they were all considered as males (Table 6).

Diagnostic alleles for identification of parental specimens

Allele frequencies were estimated in *A. pegreffii* and *A. berlandi* are reported in Suppl. Table 1. As regards 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): i.e. male specimens were considered as monoallelic, while their female counterparts were considered as biallelic. Obviously, as regards to *A. berlandi*, this consideration was not possible in monomorphic sex-linked loci (i.e. *Anisl 00314* and *Anisl 22*). Larval stages were also included in the analysis, considering the above-mentioned assignment of sex at the scored sex-linked loci.

Among the 11 SSR loci considered, *Anisl 10535*, *Anisl 05784*, *Anisl 00875*, *Anisl 7* and *Anisl 22* have shown a differential frequency distribution at some alleles in the two species (Suppl. Table 1).

From the scored genotypes, it was found that the loci *Anisl 10535, Anisl 05784*, *Anisl 00875, Anisl 7 and Anisl 22* resulted to be partially diagnostic loci between *A. pegreffii* and *A. berlandi,* i.e. they were not 100% diagnostic between the two taxa (Suppl. Table 1). Instead, the loci *Anisl 15* and *Anisl 2* loci resulted to be 100 % diagnostic between *A. pegreffii* and *A. berlandi*. In fact, at the locus *Anisl 15*, *A. berlandi* showed only the allele *235*, while *A. pegreffii* the allele *231* (Suppl. Table 1); finally, at locus *Anisl 2*, *A. berlandi* showed the allele *245*, while *A. pegreffii* showed the allele *248* (Suppl. Table 1).

The three nematodes that resulted to be heterozygous at the diagnostic site at the nuclear gene *nas10* (Figure 1), resulted to be heterozygous also at those to SSRs 100% diagnostic loci (i.e. *(i.e. Anisl 2 and Anisl 15)* Indeed, at those loci the three specimens have shown a genotype including the two fixed alternative alleles of the parental species. While, other loci, such as those partially diagnostic *SSRs loci (i.e Anisl 10535, Anisl 05784, Anisl*

00875, Anisl 7, Anisl 4 and Anisl 22) have shown in those three specimens, a genotype showing the most common alleles of the two parental species. The peaks representing the alleles found at the parental taxa and those found in the hybrid specimens, at both the 100% diagnostic and partially diagnostic loci, are reported in Fig. 3.

Multi-locus genotyping approach to discriminate* A. pegreffii *and* A. berlandi *species

Individual *Anisakis* worms were assigned to either *A. pegreffii* or *A. berlandi* based on a Bayesian clustering algorithm which is implemented in STRUCTURE 2.3.3 (Pritchard *et al.* 2000), according to their genotypes observed at the two total diagnostic SSR loci (i.e. *Anisl 15* and *Anisl 2*), at the partial diagnostic loci (i.e. *Anisl 10535, Anisl 05784* and *Anisl 00875*) and the nuclear gene *nas10* (Fig. 4). Clearly, because of the hemizygosity of the male specimens, the partial diagnostic sex-linked loci (i.e. *Anisl 7* and *Anisl 22*) were excluded from that STRUCTURE analysis, as performed in Mattiucci *et al*. (2019). Using both the highest *ln* probability and the delta-K (Evanno *et al*., 2005) optimality criteria, the STRUCTURE analysis indicated K = 2 as the clustering option which best fitted the data set (Suppl. Fig. 1).

Therefore, considering the genotype data sets inferred from five aforementioned microsatellite loci plus the *nas10* nDNA with the K=2 clustering option, all the individuals previously identified at mtDNA *cox-2* as *A. pegreffii* were assigned with high percentage of assignment to that species (>99%) (Fig. 4). Similarly, all individuals previously identified at mtDNA *cox-2* as *A. berlandi* were assigned with high percentage of assignment to that species (>99%) except for three specimens (Fig. 4). As regards the last three individuals, showing mixed ancestry between the two taxa, the analysis with STRUCTURE indicated a Q-value of those to be 0.50 (Fig. 4). As reported above, those three specimens showed heterozygous genotypes at the alternative alleles of total diagnostic SSR loci between *A. pegreffii* and *A. berlandi* (i.e. *Anisl 15* and *Anisl 2*), at the partial diagnostic SSR loci between

the two species (i.e. *Anisl 10535*, *Anisl 05784* and *Anisl 00875*) and at the diagnostic nuclear gene *nas10*. Therefore, based on these loci they are likely F1 hybrids between the two species.

For including in the Bayesian clustering analysis the partial diagnostic sex-linked loci too (i.e. *Anisl 7* and *Anisl 22*), as reported in Mattiucci *et al.* (2019), female specimens only were considered for a distinct analysis, and even then it was obtained an high percentage of assignment (>99%) to the two taxa studied (Suppl. Fig. 2). Moreover, since the three hybrid specimens (one adult and two L3 stages) showed heterozygous genotypes at the sex-linked loci, they were all females; so, they were also included in Bayesian clustering analysis with female nematodes only, and they showed an expected Q-value = 0.50 (Suppl. Fig. 2).

4.4 DISCUSSION

New diagnostic microsatellite loci between A. pegreffii *and* A. berlandi

In the present study, the utility of diagnostic SSR loci in a multilocus genotyping to identify specimens of *A. pegreffii* and *A. berlandi* in a sympatric area, has been proved. In our previosu studies (Mattiucci *et al.* 2019; Bello *et al*., 2020) a panel of eight microsatellite loci was validated to discriminate the sibling species of *A. simplex* (s. l.) complex (i.e. *A. simplex* (s. s.), *A. pegreffii*, *A. berlandi*). In particular, as regards the discrimination power of SSRs in the detection of *A. pegreffii* and *A. berlandi*, partial diagnostic loci were found at the loci *Anisl 10535, Anisl 05784, Anisl 00875* and *Anisl 7* loci (Bello *et al*.,in press). With respect to the previous SSR loci scored in those *Anisakis* species, the addition of further loci among those proposed by Mladineo (2017), some of them resulted to be of diagnostic power between the two taxa. They are the following: *Anisl 22* resulted to be a partial diagnostic, while the two others (i.e. *Anisl 15* and *Anisl 2*) resulted to be complete discriminating loci (100%). Mladineo *et al* (2017) did not analyse specimens of *A. berlandi*; therefore, the potentiality of these new markers was not possible to be detected. Instead, in the present study, three new

tools to distinguish *A. pegreffii* from *A. berlandi* are available to be used in a multilocus genotyping approach.

Hybridization between A. pegreffii *and* A. berlandi *species*

The biological species concept (BSC) (Mayr, 1963) stated that: "… the species are systems of populations: the gene exchange between these systems is limited or prevented by a reproductive isolating mechanism (RIM), i.e. by a combination of pre-zygotic and post-zygotic barriers."

Anisakis pegreffii and *A. berlandi* have always been considered as biological species, thanks to the use of genetic markers [allozymes (nDNA), ITS (rDNA), *cox-2* gene (mtDNA)], which demonstrated their genetic differentiation, in both allopatric and sympatric areas (for a review see Mattiucci *et al*., 2018a). To date, no hybridization or introgression phenomena were reported about those two taxa. Instead, in the present study, three hybrid nematodes were identified in the sympatric area of New Zealand waters, by a multilocus genotyping approach. In fact, those three specimens showed heterozygous genotypes at the two total diagnostic microsatellite loci (i.e. *Anisl 15* and *Anisl 2*), at the partial diagnostic microsatellite loci (*Anisl 10535, Anisl 05784, Anisl 00875, Anisl 7, Anisl 22,*) and at the discriminant sites of the diagnostic locus *nas10.* However, the finding of three F1 specimens does not put in doubt that *A. pegreffii* and *A. berlandi* are biological species. In fact, the presence of only three hybrids does not mean that a "stable" gene flow occurs between the two taxa, but just that sometimes the reproductive isolation mechanisms are not completely efficient. Thus, the hybridization between those two sibling species could be evaluated as a sporadic and recent phenomenon. Pre-zygotic barriers for parasites include ecological isolation, isolation by host-specificity and mate recognition systems (Southgate *et al.,* 1998). In this regard, the first two conditions could not be considered, since they were not valid about *A. pegreffii* and *A.*

berlandi species; in fact, they share both geographical area and definitive hosts (for a review see Mattiucci *et al.,* 2018a). Thus, it might perhaps be supposed that some chemical cues and/or other mating recognition systems of adult specimens could not be so efficient in a syntopic infection of the same definitive host.

The occurrence of F1 hybrids in parasite animals have already been reported in different ascaridoid species in sympatric areas: *Parascaris univalens* and *P. equorum* (Biocca *et al*., 1978; Bullini *et al*., 1978; Goday and Pimpinelli, 1986); *Ascaris lumbricoides* and *A. suum* (Criscione *et al*., 2007), *Pseudoterranova decipiens* (s. s.) and *P. krabbei* (Paggi *et al*., 1991); *Contracaecum rudolphii* sp. A and *C. rudolphii* sp. B (Mattiucci and Nascetti, unpublished results); 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); *Schistosoma mansoni* and *S. rodhaini* (Morgan *et al*., 2003; Steinauer *et al*., 2008, 2010), *Taenia saginata* and *T. asiatica* (Okamoto *et al*., 2010).

In *Anisakis simplex* (s. l.) complex, F1 hybrids between *A. simplex* (s. s.) and *A. pegreffii* species were identified. Based on a multilocus genotyping approach and by the Bayesian clustering analysis inferred from diagnostic allozyme markers and EF1 α-1 (nDNA) gene, 11 worms resulted to be as F1 hybrids between *A. simplex* (s. s.) and *A. pegreffii* species since they resulted heterozygous genotypes at all diagnostic loci there considered (Mattiucci *et al*., 2016). An interesting result emerged from that work regarded to the ITS region of rDNA, which cannot be considered as a good diagnostic between the two sibling species of parasites; in fact, one of the two discriminating positions in nucleotidic sequence has resulted to be a shared polymorphism between the two *Anisakis* taxa (Mattiucci *et al.,* 2016). No adult hybrids and backcross specimens were identified in that study. As

regards the F1 hybrids detected in the present study, two of them were at L3 larval stage, while the other one was at the adult stage. However, no backcrosses were identified in the present study. In each case, the matrilineage defined by mtDNA *cox-2* gene of those three nematodes belonged to *A. berlandi* species. The number of hybrid specimens here analyzed was too limited to make plausible assumptions about how outbreeding between those sibling taxa could occur. A more complete dataset could help to further understand this dynamics, by collecting and analyzing those two parasite species in other definitive hosts and different their sympatric areas.

As stated above, the present study emphasized once again the importance of multilocus genotyping approach as a tool for investigating and clarifying the discrimination between closely related taxa and potential hybridization phenomena. As obtained by the Bayesian clustering approach in this study, using a panel of seven microsatellite loci (two total diagnostic and five partial diagnostic) and one diagnostic nuclear marker resulted to be very useful to distinguish two sibling pure species, i.e. *A. pegreffii* and *A. berlandi*, with a very high percentage of assignment (>99%), and to identify mixed ancestry specimens (Q-value = 0.50).

Thus, investigating the hybrid parasite animals can be useful to understand whether they are able to infect new hosts in comparison to the parental species, or if they are more resistant or more susceptible to the immune system of the hosts respect to the parental species.

Laboratory outbreeding could be a useful approach to study in detail the phenotype, the behavior and the infection strategies of F1 hybrid parasites, contributing to clarify some dynamics that are difficult to observe and study in nature. Unfortunately, while for other parasite helminths the laboratory outbreeding resulted to be possible (Grabner *et al.,* 2012, Henrich *et al.,* 2013) the procedure about *Anisakis* species is more complicated. In fact, the

procedure for culture *in vitro* of L3 larval stages and adult stages was performed, but the phases of eggs hatching and the first molts of the animals were not carried out.

Utility of X-linked SSR loci for sex determination in larval stages, to evaluate allele frequencies and to perform Bayesian clustering approaches

Besides being important for population genetics studies and analysis, microsatellites resulted to be a useful and powerful tool to distinguish females from males in organisms with X0 sex determination. Sex-linked microsatellite loci are genes located on X-chromosome: in order to recognize them, it is necessary to verify if males results to be homozygous at the considered loci and if females results to be heterozygous at least at one of the same loci. If the male specimens are homozygous at a considered microsatellite locus, it means that they are not "real" homozygous, but hemizygous, because they possess only one copy of that gene, since they have the chromosome(s) inherited from the mother only, without the chromosomal paternal counterpart.

Employing a panel of microsatellite loci, Criscione *et al.,* 2007 investigated *Ascaris lumbricoides* species. The Authors obtained that the sex-linked markers should account for 20% of the genome of the studied nematode taxon.

Nevertheless, in the present study, besides the eight microsatellite loci studied in Mattiucci *et al.,* 2019 and Bello *et al.,* (*in press*), four further microsatellites were added to the analyses, and two of them resulted to be sex-linked. Thus, in this regard four sex-linked loci were discovered on a panel of eleven markers (i.e. *Anisl 00314*, *Anisl 7*, *Anisl 4* and *Anisl 22*). Therefore, 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); according to the present data set, it increased to 33%: this

represents a higher percentage (i.e. 20%) in comparison to that supposed by Criscione *et al.* (2007) in ascarids, such as *Ascaris lumbricoides*.

To date, karyotype of anisakid nematodes were not determined. The only one reference available for Ascaridida regards the nematode *Ascaris suum,* which shows 19 autosomes and 5X chromosomes (2n = 38A + 10X in females, 38A + 5X in males) (Müller and Tobler, 2000).

In the present study, it was proved that a panel of sex-linked loci could be very useful for sex-determination in organisms with not sexually mature stages during the life cycle, such as parasite animals. L3 larvae of anisakid nematodes are not reproductive, and L4 stages too are sexually immature; thus, distinguishing a female from a male in those life stages results to be impossible.

Nevertheless, if it is available a panel of sex-linked loci, that issue can be completely or partially resolved by a statistical calculation, based on expected homozygosis for adult female specimens only. In fact, starting from the expected heterozygosis obtained at sex-linked loci for female specimens (both adult and assigned to female sex by genotyping X-linked loci), it is possible to deduce the expected homozygosis at every sex-linked locus. In this way, the percentage of females showing homozygous genotypes at all sex-linked loci can be obtained from the product of expected homozygosis values calculated. Moreover, employing those X-linked loci for sex discrimination in larval stages can results to be important to understand the sex ratio of those parasites into their hosts.

In this study, the total sex ratio in the *A. pegreffii* population resulted to be pro for females (113) in comparison to the males (67), and as regards *A. berlandi* the sex ratio resulted to be pro for females (74) in comparison to the males (59) also. Considering adult and larval stages only, the sex ratio resulted to be a little more balanced: in fact, as regards *A.*

pegreffii, female larval stages were 55 *versus* 31 males, while female adults were 58 *versus* 36 males; in *A. berlandi*, female larval stages were 52 *versus* 42 males, while female adults were 22 *versus* 17 males.

In addition to the usefulness to discriminate sex in X0 sex determination species, considering a possible sex-linkage at some microsatellite loci could result to be crucial to understand why homozygous excess occurs at those considered loci. In fact, in many studies about those nuclear loci, when homozygous excess occurred, the first and main explanation for that result is the presence of null alleles, since microsatellite loci usually undergo that problem. Thus, when a microsatellite locus is assumed to be affected by null alleles, it is necessary to solely exclude that specific locus from the population genetics analyses. Therefore, a prospective important locus goes lost for the multilocus genotyping approach.

Nevertheless, if male and female specimens are available, it is deeply recommended to verify if the males are homozygous at the SSR loci affected by high observed homozygosis (also for different alleles), and if the females are heterozygous at least at one of the same loci: if sex-linkage occurs, the considered microsatellite locus cannot be employed for the analyses. In fact, keeping in mind that males are hemyzygous (that is monoallelic), while females are heterozygous (that is biallelic), allele frequencies and allele frequencies-based analyses could be performed, while as regards Bayesian clustering approach and other genotypes-based analyses could be performed on female specimens only. In the present study, *Anisl 00314*, *Anisl 7*, *Anisl 4*, *Anisl 22* resulted to be sex-linked loci in *A. pegreffii*, while only *Anisl 7* and *Anisl 4* in *A. berlandi*. Except *Anisl 00314* locus, not useful for the discrimination between *A. pegreffii* and *A. berlandi* species, *Anisl 7*, *Anisl 4* and *Anisl 22* resulted to be partial diagnostic between the two species. Thus, verifying the sex-linkage and splitting the dataset based on the

sex of studied nematodes resulted to be crucial to obtain a more reliable and informative result.

New intermediate/paratenic host record for A. berlandi *species*

Species belonging to genus *Anisakis* were known to live in all waters of the world, from the boreal hemisphere to the austral one, infecting many species of fish and cetacean hosts (Mattiucci *et al*., 2018a). Concerning that point, another result in the present study was the identification of *A. berlandi* species in a new intermediate/paratenic host, that is *Hyperoglyphe antarctica*. That result contributed to increase the knowledge of this taxon belonging to the *Anisakis simplex* (s. l.) complex; in fact, in comparison to the two other sibling species, i.e. *A. pegreffii* and *A. simplex* (s. s.), *A. berlandi* was identified in few fish hosts, generally characterized by a life cycle with both pelagic and benthonic phases.

Concluding remarks

Multilocus genotyping approach resulted to be the most powerful tool to understand and investigate complicated phenomena such as hybridization and introgression. The availability of more genetic-molecular markers to discriminate closely related taxa, in a combined approach, allows the identification with a greater reliability of the parental species and of the mixed ancestry specimens.

Furthermore, the development and employing of new markers can help to verify the real utility of the tools employed until that moment: that is, verifying their real reliability. After all, as regards genus *Anisakis*, a multilocus genotyping approach based on allozymes and the nuclear gene EF1 α-1 allowed to clarify that one nucleotidic site (i.e. 294 bp) at ITS region of rDNA cannot be considered as diagnostic site between *A. simplex* (s. s.) and *A.*

pegreffii species (Mattiucci *et al*., 2016). In fact, actually it consisted in a share polymorphism between the two taxa (Mattiucci *et al*., 2016).

If the combination of more nuclear markers had not been performed, the ITS region would still be considered as a reliable discriminating marker about the two sibling species belonging to the *A. simplex* (s. l.) complex.

Unfortunately, since 2016, other studies have employed the ITS region of rDNA as tool to identify hybrids between *A. simplex* (s. s.) and *A. pegreffii* (Costa *et al*., 2016, Gazzonis *et al*., 2017, Mladineo *et al*., 2017a), but its use should be severely restricted to avoid misleading results.

In the present study, a new panel of eleven nuclear genetic markers was confirmed to be very powerful to distinguish parental species and their F1 hybrids. In this work, it was not possible to analyse the allozyme loci, because the samples were stored in alcohol the sequences analysis at the EF1 α-1 nDNA was not employed, because it shows a partial amplification in *A. berlandi* species; thus primers for the species *A. berlandi* need to be newly designed (Mattiucci *et al*., data not published).

In future works, a multilocus genotyping approach, consisting of microsatellites, allozymes, *nas10* and SNPs at other gene loci, could be performed. By a multilocus genotyping approach, it could be possible to study other sympatric areas between *A. berlandi* and *A. pegreffii* (i.e. South African coast, Argentine coast, (Mattiucci *et al*., 2018a). Moreover, it could be possible to study the other sibling species belonging to *A. simplex* (s. l.) complex, i.e. *A. simplex* (s. s.), in the sympatric areas with *A. pegreffii* (i.e. Iberian Atlantic coasts and Japanese coasts) and with *A. berlandi* (i.e. Canadian and Californian waters).

Finally, if some of the genetic-molecular markers employed for the analyses shows a sex-linkage, can result to be very useful to distinguish sex in larval stages not sexually mature. In fact, in the present study, the availability of a panel of X-linked loci allowed to determinate sex in L3 and L4 larval stages of the two taxa considered, allowing to perform genetic analyses such as allele frequencies, allele frequencies-based approaches and Bayesian clustering approach (considering only female specimens) too, keeping in mind of the hemizygosity of male specimens.

4.5 TABLES AND FIGURES

Table 1. Host species, life-history stage and sex of adult specimens of the nematodes here studied from New Zealand coast, belonging to *Anisakis simplex* (s. l.) complex.

Host species	N	N_A ♂♂	N_A ♀♀	N_{L4}	N_{L3}
Cephalorhynchus hectori	3	1	2	-	-
Globicephala melas	191	52	79	60	-
Grampus griseus	3	-	-	3	-
Allocytus sp.	9	-	-	-	9
Hyperoglyphe antarctica	9	-	-	-	9
Hoplostethus atlanticus	24	-	-	-	24
Macruronus novaezelandiae	25	-	-	-	25
Mora moro	19	-	-	-	19
Pseudophycis bachus	12	-	-	-	12
Rexea solandri	10	-	-	-	10
Trachurus declivis	11	-	-	-	11
Tot:	*316*	*53*	*81*	*31*	*151*

Table 2. Assignment to the parental taxa, i.e. *Anisakis pegreffii* (AP) and *A. berlandi* (AB), or mixed ancestry (He), of nematode specimens, according to their genotypes observed at mitochondrial (i.e. *cox-2* gene) or nuclear (i.e. SSR loci and *nas10* gene) markers used in the present study.

Host species	N	mtDNA		nDNA					
		cox-2		SSRs			*nas10*		
		AP	AB	AP	AB	He	AP	AB	He
Cephalorhynchus hectori	3	3	-	3	-	-	3	-	-
Globicephala melas	191	137	53	137	53	1	137	53	1
Grampus griseus	3	3	-	3	-	-	3	-	-
Allocytus 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	-	9	-	2	9	-	2
Tot:	*316*	*180*	*133*	*180*	*133*	*3*	*180*	*133*	*3*

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 because of monomorphism in both species of *Anisakis* about the latter loci. *N*= number of genotyped specimens at each locus; H_o= observed heterozygosity; H_e= expected heterozygosity; A= number of alleles detected at each locus. *P* indicates the significance ($P < 0.05$) of the deviation from HWE expectation. ***$P \ll 0.001$.

		A. pegreffii	*A. berlandi*
Locus			
Anisl 00185	N	180	133
	H_o	0.77	0.76
	H_e	0.81	0.84
	P value	0.45	0.23
	A	8	11
Anisl 00314	N	180	133
	H_o	0.42	-
	H_e	0.75	-
	P value	***	-
	A	9	1
Anisl 10535	N	180	133
	H_o	0.71	0.01
	H_e	0.77	0.01
	P value	0.54	1.00
	A	10	2
Anisl 05784	N	180	133
	H_o	0.81	0.04
	H_e	0.78	0.04
	P value	0.21	1.00
	A	13	2
Anisl 08059	N	180	133
	H_o	0.83	0.35
	H_e	0.86	0.30
	P value	0.11	0.36
	A	14	4
Anisl 00875	N	180	133
	H_o	0.44	0.10
	H_e	0.48	0.09
	P value	0.53	1.00
	A	9	5
Anisl 7	N	180	133
	H_o	0.20	0.51
	H_e	0.27	0.92
	P value	***	***
	A	4	28
Anisl 4	N	180	133
	H_o	0.50	0.38
	H_e	0.82	0.84
	P value	***	***
	A	14	16
Anisl 22	N	180	133
	H_o	0.38	-
	H_e	0.60	-
	P value	***	-
	A	7	1

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 only. *N*= number of genotyped specimens at each locus; H_o= observed heterozygosity; H_e= expected heterozygosity. *P* indicates the significance ($P < 0.05$) of the deviation from HWE expectation. ***$P \ll 0.001$.

		A. pegreffii	*A. berlandi*
Locus			
Anisl 00314	N	58	-
	H_o	0.65	-
	H_e	0.77	-
	P value	0.11	-
Anisl 7	N	58	22
	H_o	0.31	1.00
	H_e	0.32	0.87
	P value	0.67	0.51
Anisl 4	N	58	22
	H_o	0.79	0.77
	H_e	0.84	0.87
	P value	0.71	0.60
Anisl 22	N	58	-
	H_o	0.59	-
	H_e	0.60	-
	P value	0.28	-

Table 5. Genetic diversity at the four sex-linked loci (i.e. *Anisl 00314*, *Anisl 7*, *Anisl 4*, *Anisl 22*) in *A. pegreffii* and *A. berlandi* species, estimated in female specimens, both adult and larval stages assigned to female sex from genotypes at sex-linked loci above-mentioned. *N*= number of genotyped specimens at each locus; H_o= observed heterozygosity; H_e= expected heterozygosity. *P* indicates the significance ($P < 0.05$) of the deviation from HWE expectation. ***$P \ll 0.001$.

		A. pegreffii	*A. berlandi*
Locus			
Anisl 00314	N	113	-
	H_o	0.67	-
	H_e	0.75	-
	P value	0.06	-
Anisl 7	N	113	74
	H_o	0.32	0.97
	H_e	0.33	0.92
	P value	0.74	0.70
Anisl 4	N	113	74
	H_o	0.80	0.68
	H_e	0.82	0.83
	P value	0.84	0.17
Anisl 22	N	113	-
	H_o	0.61	-
	H_e	0.59	-
	P value	0.30	-

Table 6. Assignment to female or male sex of larval stages (L4/L3) belonging to *Anisakis pegreffii* and *A. berlandi* species here studied, according to observed genotypes at four sex-linked loci (i.e. *Anisl 00314*, *Anisl 7*, *Anisl 4*, *Anisl 22*) in *A. pegreffii*, and at two sex-linked loci (i.e. *Anisl 7* and *Anisl 4*) in *A.* berlandi.

Parasite species		$N_{L4/L3}$	$N_{L4/L3}$ ♀♀	$N_{L4/L3}$ ♂♂
A. pegreffii	*Globicephala melas*	46	33	13
	Grampus griseus	3	3	-
	Macruronus novaezelandiae	3	1	2
	Mora moro	8	4	4
	Pseudophycis bachus	9	5	4
	Rexea solandri	8	5	3
	Trachurus declivis	9	4	5
	Tot:	86	55	31
A. berlandi	*Globicephala melas*	14	9	5
	Allocytus sp.	9	7	2
	Hyperoglyphe antarctica	9	5	4
	Hoplostethus atlanticus	24	15	9
	Macruronus novaezelandiae	22	11	11
	Mora moro	11	4	7
	Pseudophycis bachus	3	-	3
	Rexea solandri	2	1	1
	Tot:	94	52	42

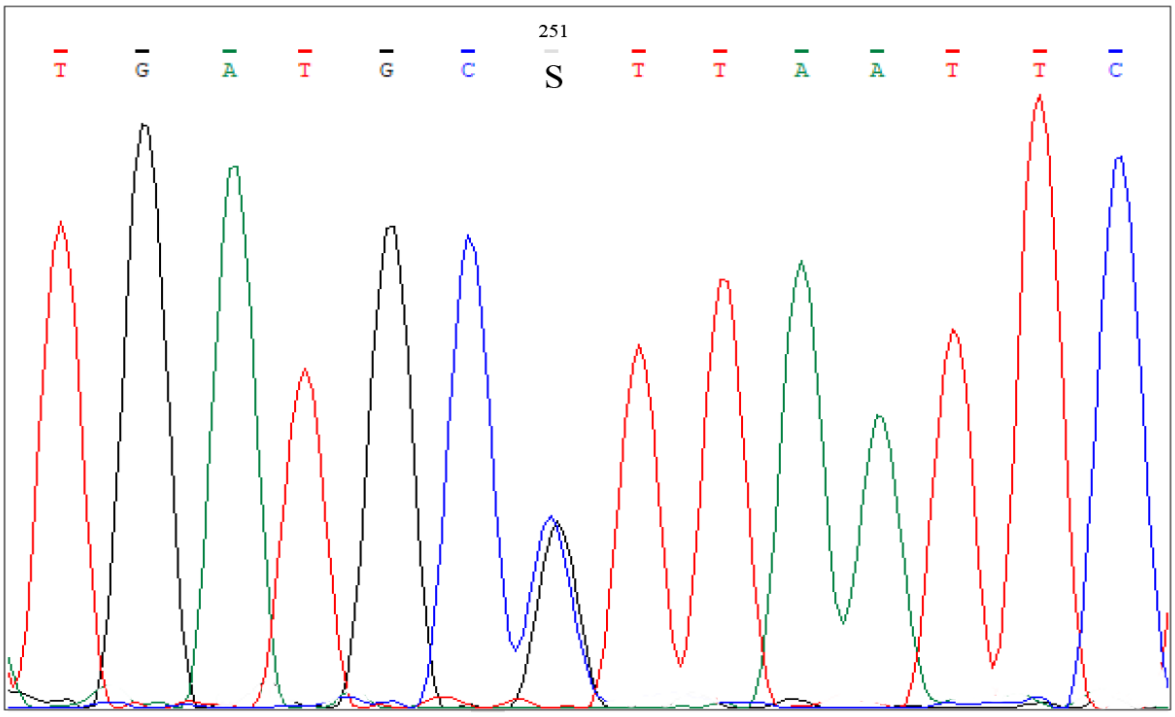


Figure 1. Heterozygote pattern at the nucleotide diagnostic position between *A. pegreffii* and *A. berlandi* species of the *nas10* (nDNA). Standard IUPAC codes were used, i.e. S = C/G.

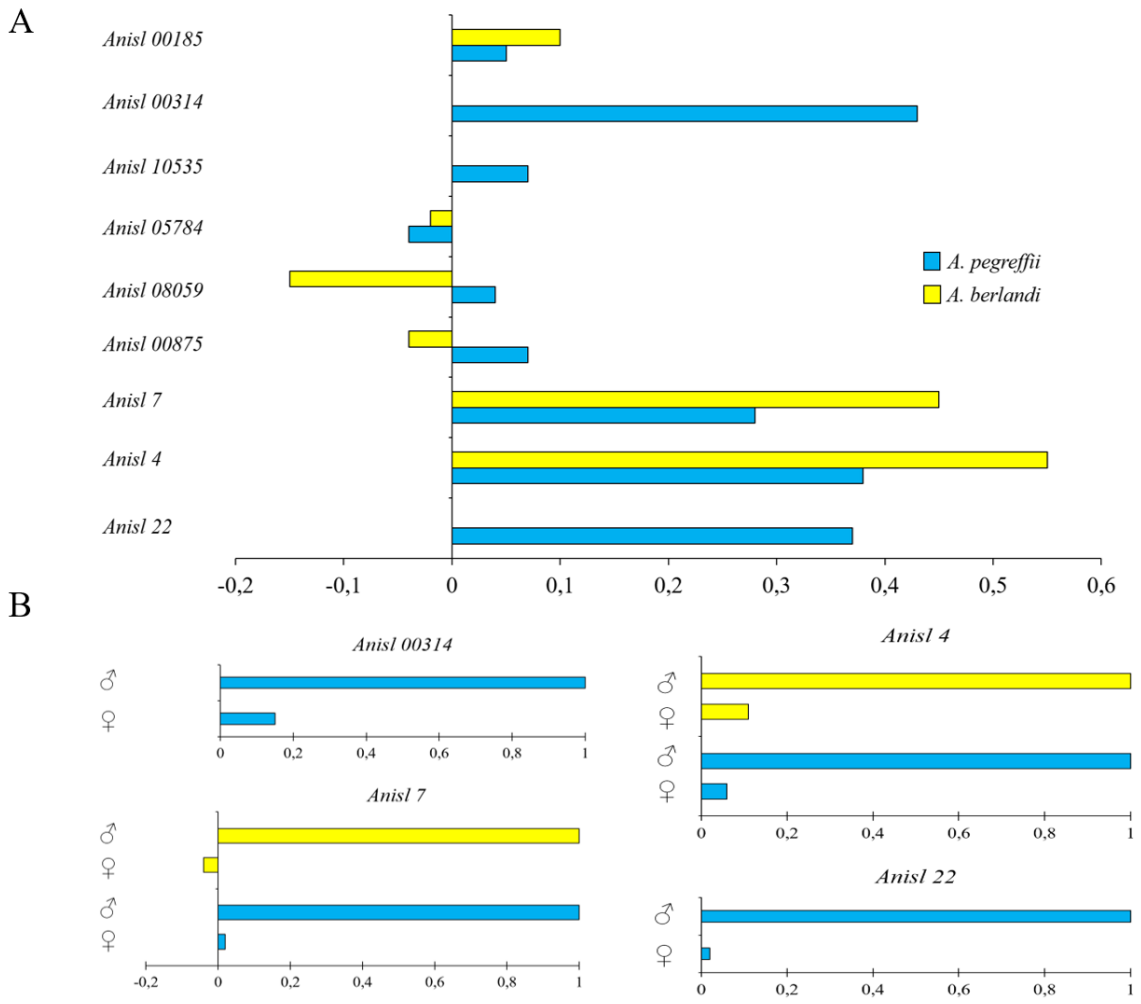


Figure 2. (A) F_{IS} calculated at nine microsatellite loci (except *Anisl 15* and *Anisl 2*, because of their monomorphism) studied in the two species *A. pegreffii* and *A. berlandi*; (B) F_{IS} in male and female specimens of *A. pegreffii* and *A. berlandi*, at the four sex-linked loci for the first species (i.e. *Anisl 00314*, *Anisl 7*, *Anisl 4* and *Anisl 22*) and at the two sex-linked for the latter species (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).

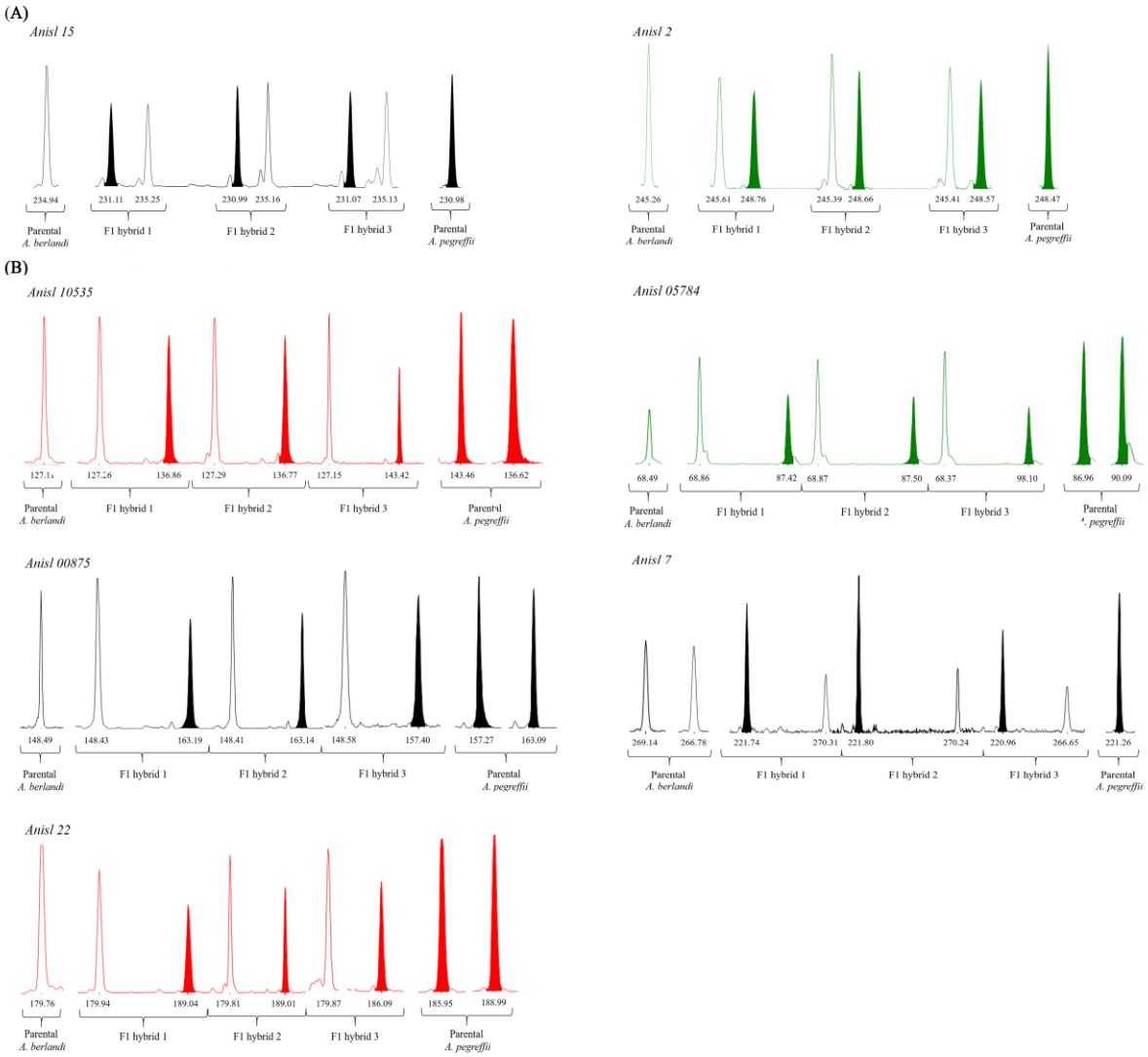


Figure 3. Electropherograms and observed genotypes of pure *A. berlandi* (on the left), F1 hybrids (in the middle) and pure *A. pegreffii* (on the right). (A) Observed genotypes at total diagnostic loci (100%) between species (i.e. *Anisl 15* and *Anisl 2*) (B) Observed genotypes at the partial diagnostic SSR loci (i.e. *Anisl 10535*, *Anisl 05784*, *Anisl 00875*, *Anisl 7* and *Anisl 22*). Discriminant alleles for species *A. berlandi* were reported as empty peaks; discriminant alleles for species *A. pegreffii* were reported as full peaks.

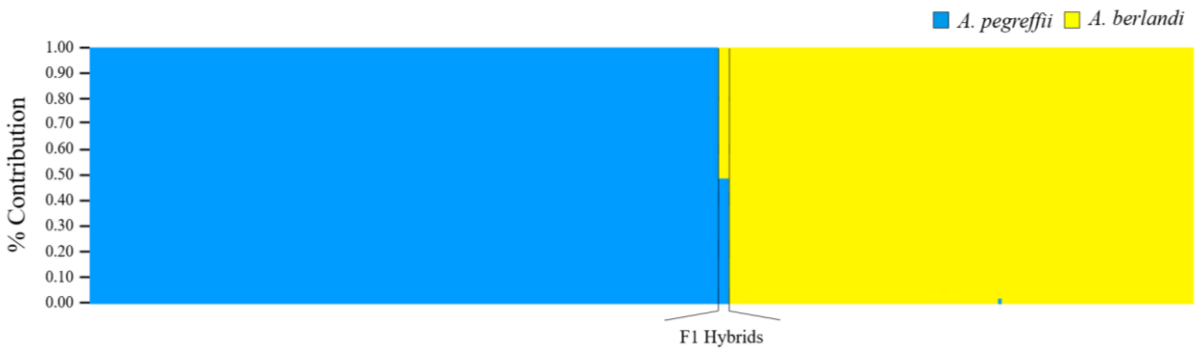


Figure 4. Percentage contribution (Q value) of *A. pegreffii* and *A. berlandi* species to the multilocus genotype of each studied individual (barplot), estimated at five SSRs loci (i.e. *Anisl 10535, Anisl 05784, Anisl 00875, Anisl 15* and *Anisl 2*) and at the nuclear gene *nas10*, by using STRUCTURE with k = 2.

Supplementary Table 1. Allele frequencies observed at eleven microsatellite loci of *A. pegreffii* and *A. berlandi* populations 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. As regards sex-linked loci, all specimens, both adult and larval stages assigned to female or male sex from genotypes at sex-linked loci, were considered.

Locus	Alleles	*A. pegreffii*	*A. berlandi*
Anisl 00185	N	180	133
	182	-	0.01
	185	-	0.01
	188	0.02	0.02
	191	0.05	0.11
	194	0.22	0.16
	197	0.22	0.20
	200	0.19	0.24
	203	0.24	0.11
	206	0.04	0.10
	209	0.02	0.02
	212	-	0.02
Anisl 00314	N	180	133
	96	0.04	-
	100	0.26	1.00
	104	0.32	-
	108	0.27	-
	112	0.07	-
	116	0.01	-
	120	0.01	-
	124	0.01	-
	128	0.01	-
Anisl 10535	N	180	133
	125	0.01	-
	128	0.01	0.99
	131	0.01	0.01
	134	0.19	-
	137	0.20	-
	140	0.26	-
	143	0.28	-
	146	0.02	-
	149	0.01	-
	152	0.01	-

(continues)

(continued)

Locus	Alleles	*A. pegreffii*	*A. berlandi*
Anisl 05784	N	180	133
	66	0.01	-
	69	0.01	0.98
	72	0.01	0.02
	75	0.02	-
	78	0.05	-
	81	0.03	-
	84	0.08	-
	87	0.24	-
	90	0.34	-
	93	0.14	-
	96	0.05	-
	99	0.01	-
	102	0.01	-
Anisl 08059	N	180	133
	82	0.02	0.01
	86	0.19	0.82
	90	0.05	0.16
	94	0.14	0.01
	98	0.16	-
	102	0.19	-
	106	0.10	-
	110	0.04	-
	114	0.04	-
	118	0.01	-
	122	0.02	-
	126	0.02	-
	130	0.01	-
	134	0.01	-
Anisl 00875	N	180	133
	142	-	0.01
	145	0.01	0.01
	148	0.01	0.94
	151	0.05	0.03
	154	0.03	0.01
	157	0.71	-
	160	0.13	-
	163	0.04	-
	166	0.01	-
	169	0.01	-

(continues)

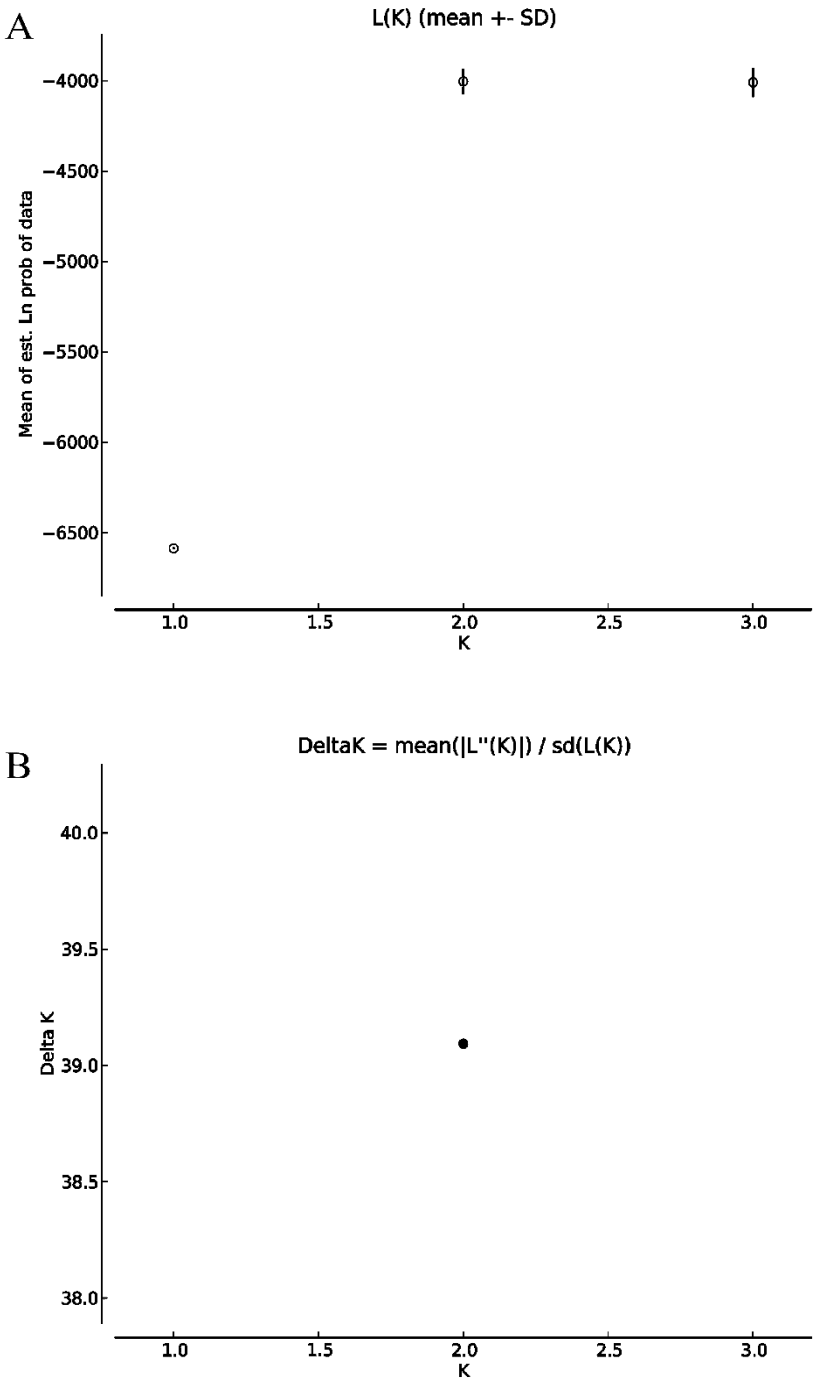
(continued)

Locus	Alleles	*A. pegreffii*	*A. berlandi*
Anisl 7	N	180	133
	216	0.02	-
	219	0.07	-
	222	0.83	-
	225	0.08	0.01
	228	-	0.01
	237	-	0.01
	240	-	0.01
	243	-	0.01
	246	-	0.01
	249	-	0.01
	252	-	0.01
	258	-	0.03
	261	-	0.05
	264	-	0.06
	267	-	0.14
	270	-	0.15
	273	-	0.16
	276	-	0.05
	279	-	0.05
	282	-	0.07
	285	-	0.02
	288	-	0.05
	291	-	0.01
	294	-	0.01
	297	-	0.01
	300	-	0.01
	303	-	0.01
	306	-	0.01
	309	-	0.01
	324	-	0.01
	345	-	0.01
Anisl 4	N	180	133
	124	-	0.01
	127	0.03	0.16
	130	0.04	0.01
	133	0.31	0.01
	136	0.13	0.01
	139	0.17	0.37
	142	0.14	0.10
	145	0.07	0.05
	148	0.03	-
	151	0.02	0.02
	154	0.02	0.01
	157	0.01	0.06
	160	-	0.11
	163	-	0.04
	166	-	0.01
	169	0.01	0.01
	172	0.01	0.02
	175	0.01	-

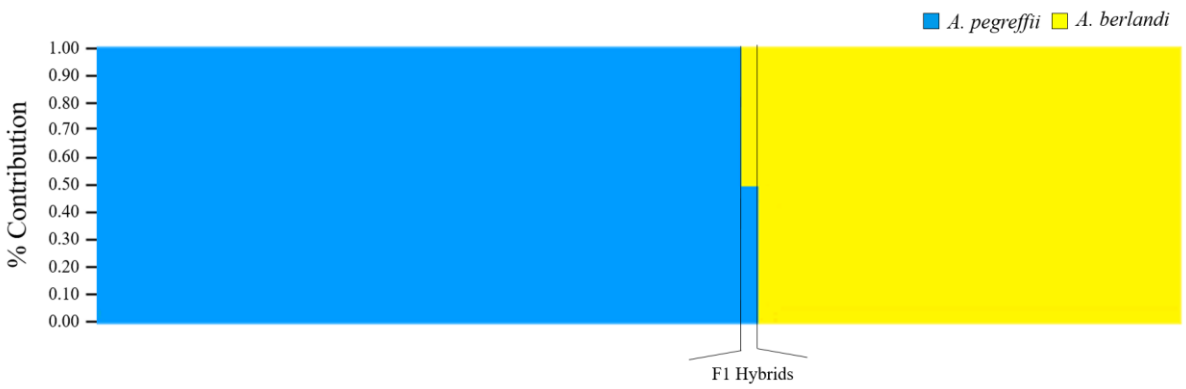
(continues)

(continued)

Locus	Alleles	*A. pegreffii*	*A. berlandi*
Anisl 22	N	180	133
	179	0.01	1.00
	182	0.04	-
	185	0.49	-
	188	0.39	-
	191	0.05	-
	194	0.01	-
	197	0.01	-
Anisl 15	N	180	133
	231	1.00	-
	235	-	1.00
Anisl 2	N	180	133
	245	-	1.00
	248	1.00	-



Supplementary Figure 1: Estimate of the best number of clusters (K) describing the population structure of the *Anisakis* spp. samples analyzed in the present study. The computation was carried out by using STRUCTURE HARVESTER. (A) highest ln-probability and (B) DeltaK optimality criteria.



Suppplementary Figure 2. Percentage contribution (Q value) of *A. pegreffii* and *A. berlandi* species to the multilocus genotype of each studied individual (barplot), only considering adult and assigned female specimens. The analysis was estimated at five SSRs loci (i.e., *Anisl 10535*, *Anisl 05784*, *Anisl 00875*, *Anisl 15* and *Anisl 2*), plus at two sex-linked loci (i.e. *Anisl 7* and *Anisl 22*) and at the nuclear gene *nas10*, by using STRUCTURE with k = 2.

5. CONCLUDING REMARKS

5.1 SUMMARY OF RESULTS

In this Thesis's work, the results achieved by the employment of microsatellite loci (SSRs loci), as a genetic tool in the recognition of the species of the *A. simplex* (s. l.), are reported. A panel of novel seven SSR loci was developed on *A. pegreffii* species for this work (Mattiucci *et al.,* 2019). Then, the cross-amplification on the species *A. simplex* (s. s.) (Mattiucci *et al.,* 2019) and *A. berlandi* (Bello *et al*., 2020) was validated. Further five SSR loci developed by Mladineo *et al.* (2017) were also added for studying the genetic structure of *A. simplex* (s. l.) complex. The data obtained from the analysis by microsatellite loci showed the presence of diagnostic alleles among the three species (i. e. *Anisl 10535, Anisl 05784, Anisl 00875, Anisl 7, Anisl 4, Anisl 22, Anisl 15, Anisl 2*) (Mattiucci *et al.,* 2019; Bello *et al*., 2020).

The results obtained by this approach furtherly supported that the three sibling species belonging to *A. simplex* (s. l.) complex (i.e. *A. simplex* (s. s.), *A. pegreffii* and *A. berlandi*), are closely related taxa, but clearly distinct species. The genetic distances (D_c, Cavalli-Sforza and Edwards, 1967) obtained from allele frequencies calculated at those SSR loci and UPGMA tree showed a clear genetic differentiation of the three sibling species, at both inter- and intra-specific level (Mattiucci *et al*., 2019; Bello *et al*., 2020). Those results were concordant with those previously achieved by using both mitochondrial and nuclear markers (Mattiucci *et al*., 2014). Finally, Bayesian clustering approach, performed using datasets from the SSR loci, proved how these novel nuclear loci can be considered a powerful tool to discriminate the three species belonging to *A. simplex* (s. l.) complex (Mattiucci *et al.,* 2019; Bello *et al*., 2020).

Additionally, the employment of this panel of nuclear markers, *plus* other diagnostic genetic markers, such as two nuclear gene loci recently discovered and validated, i.e. EF1 α-1

(Mattiucci *et al*., 2016) and *nas10* (nDNA) (Palomba *et al*., submitted), allowed to increase the power of the identification of specimens belonging to the three species of the *A. simplex* (s. l.) complex. This multilocus genotyping approach permitted to recognize parental taxa, as well as their mixed ancestry. In fact, for the first time, the recognition of F1 hybrids between *A. pegreffii* and *A. berlandi* was detected in the sympatric area of the New Zealand waters (Bello *et al*., *in prep*).

To date, the multilocus genotyping approach to discover hybridization events in *A. simplex* (s. l.) complex were carried out between *A. simplex* (s. s.) and *A. pegreffii* species: (Mattiucci *et al*., 2016). Thus, the application of this genetic approach will be valuable to evidence the occurrence of hybridizationa and/or introgression phenomena between the species of this complex in their sympatric areas.

Another important contribution provided by the application of SSR loci is the finding of sex-linked ones in the *A. simplex* (s. l.) complex. Five SSR loci resulted to be sex-linked in the three species of the *A. simplex* (s. l.) complex. They are: *Anisl 00314, Anisl 7, Anisl 4* , *Anisl 15* and *Anisl 22* (Mattiucci *et al.*, 2019; Bello *et al*., 2020, *in prep,* and data not shown). This finding showed so far how a panel of X-linked loci could be useful to sex determination in the larval stages of the species *A. pegreffii* and *A. berlandi* (Bello *et al*., in prep). However, among those SSR loci, in the case of *A. pegreffii* and *A. simplex* (s. s.) four loci can be used (*Anisl 00314* in *A. simplex* (s. s.) cannot be considered because of null alleles). While, in the case of *A. berlandi* only two of them (i. e. *Anisl 7* and *Anisl 4*) are useful in the contest, because the remainings resulted monomorphic in this taxon (Study 3).

5.2 FUTURE PERSPECTIVES

Diagnostic power of SSR loci to distinguish the sibling species of A. simplex *(s. l.) complex*

The genetic investigation by using SSR loci can be extended in other basin waters and hosts included in the range of distribution of the three species, i.e. *A. simplex* (s. s.), *A. pegreffii* and *A. berlandi*. This approach will also gather further information about the population genetic structure of the three species, out of that so far discovered (Mattiucci *et al*., 2018, 2019). For instance, it could be interesting investigate if allele frequencies are conserved in the whole range of distribution, or if some populations resulted to be differentiated.

Furthermore, the diagnostic power of SSR loci, *plus* the application of other nuclear loci so far discovered, will be useful for detecting and understanding the occurrence of hybridization phenomena in other sympatric areas of the three taxa belonging to *A. simplex* (s. l.) complex (Mattiucci *et al*., 2018).

As regards the evidence of sex-linkage observed in some SSR loci, it could be useful tool to provide the sex-ratio of those parasite species into their hosts, both intermediate/paratenic and definitive ones. Therefore, the sex-ratio detected by genetic methods would allow to suppose if it is involved in the occurrence of hybridization events between the three species, as previously hypothesized, for instance, between *A. pegreffii* and *A. simplex* (s. s.) (Mattiucci *et al*. 2016).

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