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 the most widespread groups of ascaridoid nematodes in the marine ecosystem. Three closely related taxa are recognized 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 microsatellites loci (*Anisl 05784*, *Anisl 08059*, *Anisl 00875*, *Anisl 07132, Anisl 00314, Anisl 10535, 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; *iv)* estimate levels of genetic differentiation at both the inter- and intra-specific level.

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

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 by 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 characterised 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; Buchman and Merdhana, 2016; Mattiucci *et al.,* 2018a; Guardone *et al*., 2018). 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 fish-borne 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 recognized 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 SSRs (Simple Sequence Repeats), 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; *iv)* infer data on their population genetic structure.

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 gryseus* (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 X100–400 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 cetyltrithylammonium bromide method (CTAB) was used as 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^{TM}) (Sambrook and Russell, 2001). First, aliquots with a standard concentration of 10 ng/ml 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 sec (denaturation), 58°C for 40 sec (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 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*., 2016), for the EF1 α1 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 1X solution, for 72 h (the PBS 1X 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-labeled GTAT, GATA, repeat oligonucleotides. The SSRs-enriched library was analyzed 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 7 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 analyzed 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 analyzed with the novel panel of SSRs markers.

PCR amplification of SSRs markers, and genotyping

Two Multiplex PCR amplifications (with 4 and 3 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 10µM labeled 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 30s, 56°C for 90s, and 72°C for 60s. 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 7 loci selected, i.e. the repeat motif, the bp size, the primer sequences and its labeling 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 labeled 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 at 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 heterozigosity (*Ho*), the expected heterozigosity (*He*), 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 (*Fis*) from the genetic data sets obtained at the SSRs loci in the analyzed populations of the two *Anisakis* species, was estimated using ARLEQUIN version 3.5 (Excoffier and Lischer 2010), and *Fst* (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 analyzed 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 100000 MCMC iterations, following a burn-in of 50000 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).

Results

Identification of Anisakis *spp. specimens by sequencing of* 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 analyzed. 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 analyzed 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 analyzed 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 (*He)* (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 (*Ho*) and expected *(He*) heterozygosity resulted at loci *Anisl 10535, Anisl 07132, Anisl 05784, Anisl 08059* and *Anisl 00875*, in all the analyzed 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 *Ho* and *He* 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 microsatellites loci

Statistically high significant departures from the HWE in all the analyzed 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 *Stenella coeruleoalba* from Adriatic Sea (AD), in *Globicephala melas* and

Cephalorhynchus hectori from New Zealand waters, as well as adult worms of *A. simplex* (s. s.) recovered in *Phocaena 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 8 different alleles in populations of *A. simplex* (s. s.); it resulted to be less polymorphic in *A. pegreffii*, showing up to 4 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 microsatellites 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 analyzed 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 *SSRs* 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 hemizigosity 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 *Fst* values were calculated for all the population pairs of the two *Anisakis* species, to quantify the extent of genetic sub-structuring 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 *Fst* ≈ 0.29 $p<<0.001$) (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 analyzing 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 *versus* adults from fish and cetacean hosts being, *Fst*≈ 0.008 and *Fst*≈ 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, *Fst*≈ 0.008. The highest *Fst* value= 0.021 ($p<0.001$) was found between the Baltic Sea (BA) population of *A. simplex* (s. s.) *versus* 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 *versus* AD, *Fst*= 0.002), or between the austral populations of *A. pegreffii* from off the New Zealand and Argentine coast (*Fst*= 0.007) (Table 6). Whereas, higher significant genetic sub-structuring (on average, *Fst*≈

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 analyzed 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) *versus 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 six microsatellite loci (Table 5). Both analyses agreed in depicting a high level of genetic divergence between the analyzed 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 sub-structuring 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).

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, microsatellite 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 (Criscione *et al*., 2007; Johnson *et al.,* 2006; Redman *et al.*, 2008; Temperley *et al.*, 2009; Betson *et al.,* 2011; Patrelle *et al.,* 2014; Reid *et al.,* 2012; Rabelo *et al.,* 2017; Greef *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 (Criscione *et al.,* 2007; Redman *et al.,* 2008; Grillo *et al*., 2006, 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 Table 4a,b) was taken into account, excluding those loci when population genetic data sets of that species were being analyzed.

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* SSRs 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) (Muller 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 (Muller 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 the 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 Anisakis 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 Anisakis 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 microsatellites loci, it was demonstrated that the populations analyzed 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, *Fst*= 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 allozyme level: indeed, in *A. pegreffii* a *Gst*≈ 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 *cox2* 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 *Fst* values observed at the 5 SSRs loci, the species *A. pegreffii* showed high gene flow value (*Nm*= 7.8) between the analyzed 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 analyzed 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 *Fst* values observed between the studied populations of *A. simplex* (s. s.), revealed a low level of genetic sub-structuring among the analyzed 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.) analyzed 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; 2018). 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 hosts 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 analyzing 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 *Anisakis simplex* (s. l.) complex.

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Conflict of interest

None.

Ethical Standards

We declare that there are no ethical issues related to this paper. We have not used laboratory animals.

We confirm that for vertebrate animals (dead animals) examined for parasites, we have followed the standard principles defined by the European Convention for the Protection of Vertebrate Animals.

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Table 1. Number of specimens of *Anisakis pegreffii* and *A. simplex* (s. s.) analyzed at the 8 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 4th and 3rd larval stages, respectively.

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	NZ	*Cephalorhynchus hectori*	3	1	2	-	-	3	3	3
	New Zealand coast		*Globicephala melas*	158	36	50	57	-	158	135	158
	(44°30'S - 172°58'E)		*Grampus griseus*	3	-	-	3	-	3	2	3
	SW Atlantic Ocean	AR	*Macruronus magellanicus*	46	-	-	-	46	46	45	46
	Argentina coast		*Merluccius hubbsi*	31	-	-	-	31	31	31	31
	(41°30'S - 64°15'W)										
	Mediterranean Sea	TY	*Engraulis encrasicolus*	1	-	-	-	1	1	1	1
	Tyrrhenian Sea		*Lepidopus caudatus*	29	-	-	-	29	29	28	29
	(41°7'N - 13°24'E)		*Lophius budegassa*	2	-	-	-	2	2	2	2
			Merluccius merluccius	39	-	-	-	39	39	33	39
			Scomber scombrus	13	-	-	-	13	13	10	13
	Western Adriatic Sea	AD	*Stenella coeruleoalba*	58	22	25	11	-	58	45	58
	(42°18'N - 15°35'E)		*Lophius piscatorius*	3	-	-	-	3	3	3	3
			Merluccius merluccius	38	-	-	-	38	38	27	38
			Scomber scombrus	7	-	-	-	7	7	5	7
			Trachurus trachurus	20		-		20	20	2	20
				Tot 451					*451*	*372*	*451*
Anisakis simplex (s. s.)	NE Atlantic Ocean	GS	*Merluccius merluccius*	115	-	-	-	115	115	70	115
	Grand Sole Bank										
	(49°38'N - 10°10'W)										
	English Channel	EC	*Phocoena phocoena*	79	45	34	-	-	79	77	79
	(48°38' N - 4°34'W)		*Clupea harengus*	97		-	-	97	97	54	97
	North Sea	NS	*Clupea harengus*	67	-	-	-	67	67	50	67
	(59°13'N - 00°14'W)										

Norwegian Sea (68º52'N - 3º08'E)	NW	*Clupea harengus*	41	-	-	-	41	41	39	41
		Micromesistius poutassou	10	-	-	-	10	10	-	10
		Scomber scombrus	26	-	-	-	26	26	8	26
Baltic Sea (58º29'N - 19º51'E)	BA	*Clupea harengus*	57	-	-	-	57	57	43	57
			Tot 492					*492*	*341*	*492*

Table 2. Locus name, primers 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.) analyzed in the present study. For population codes 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
Anisl 00185	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)
Anisl 00314	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)
Anisl 10535	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)
Anisl 07132	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)
Anisl 05784	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)
Anisl 08059	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)
Anisl 00875	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)
Anisl 7	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. *Anisl 00314* and *Anisl 7*) 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
	H_o	0.67	0.84	0.24
	H_e	0.77	0.77	0.80
	p-value	n.s.	n.s.	***
Anisl 7	N	52	25	34
	H_o	0.19	0.67	0.67
	H_e	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. As regards 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	-	-	-	-	-	-

(continued)

(continues)

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	-	-	-	-	-	-	-	-
	252	-	-	-	-	-	0.13	-	-	-
	255	-	-	-	-	-	0.48	-	-	-
	258	-	-	-	-	-	0.18	-	-	-
	261	-	-	-	-	-	0.11	-	-	-
	264	-	-	-	-	-	0.04	-	-	-
	267	-	-	-	-	-	0.03	-	-	-
	270	-	-	-	-	-	0.02	-	-	-
	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* (See Table 1, Fig.1) (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	0.002	-	0.017
BA	0.226**	0.271**	0.296**	0.307**	0.003	0.004	0.021*	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. * indicates alleles found in larval samples of *A. simplex* (s. s.); they are not reported in Table 5, because the estimation of the allele frequencies was calculated at the sex-linked locus *Anisl 7* only on adult (female and male) specimens.

	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*		

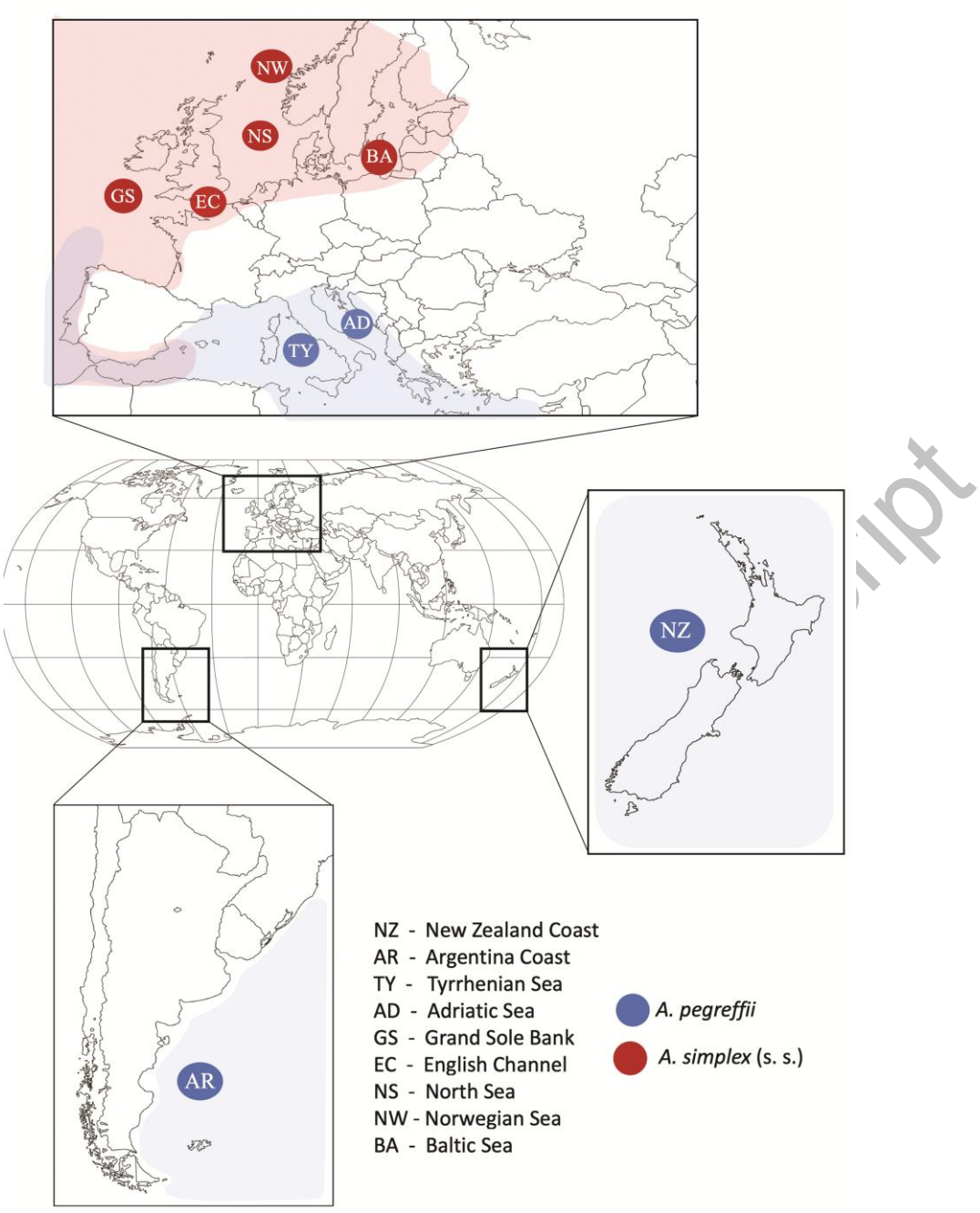

Fig. 1. Collecting sites of populations of *A. simplex* (s. s.) and *A. pegreffii* analyzed in the present study, mapped into the geographical range of the two parasites species (For location reference codes see Table 1).

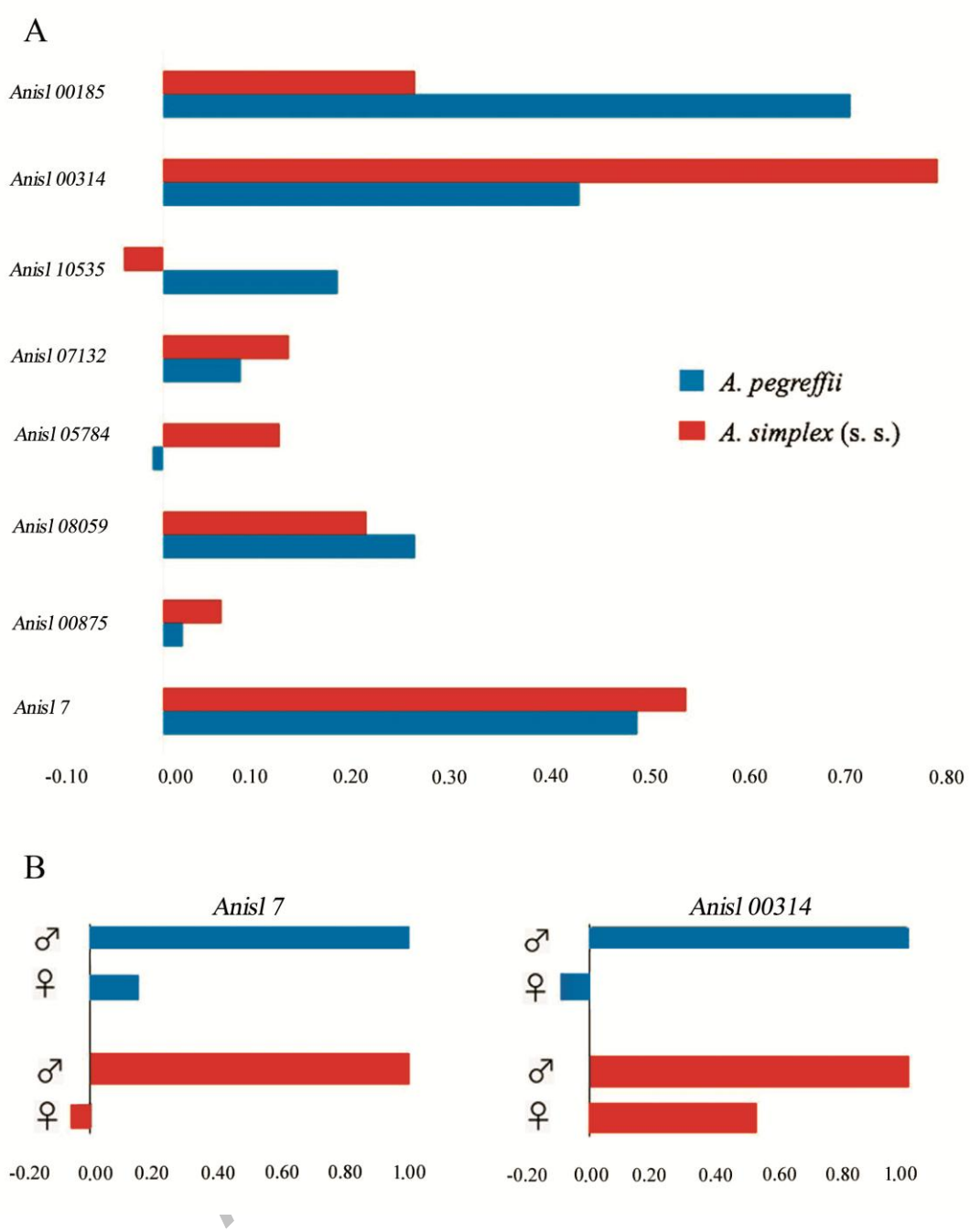

Fig. 2. A) F_{IS} calculated at the eight microsatellites loci studied in the two species *Anisakis pegreffii* and *A. simplex* (s. s.). Negative values indicate heterozygous excess while positive values indicate homozygous excess from that expected under Hardy-Weinberg Equilibrium (HWE); B) F_{IS} in 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*.

Fig. 3. Percentage contribution (Q value) of *Anisakis 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 following: 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; AR: Argentine coast.

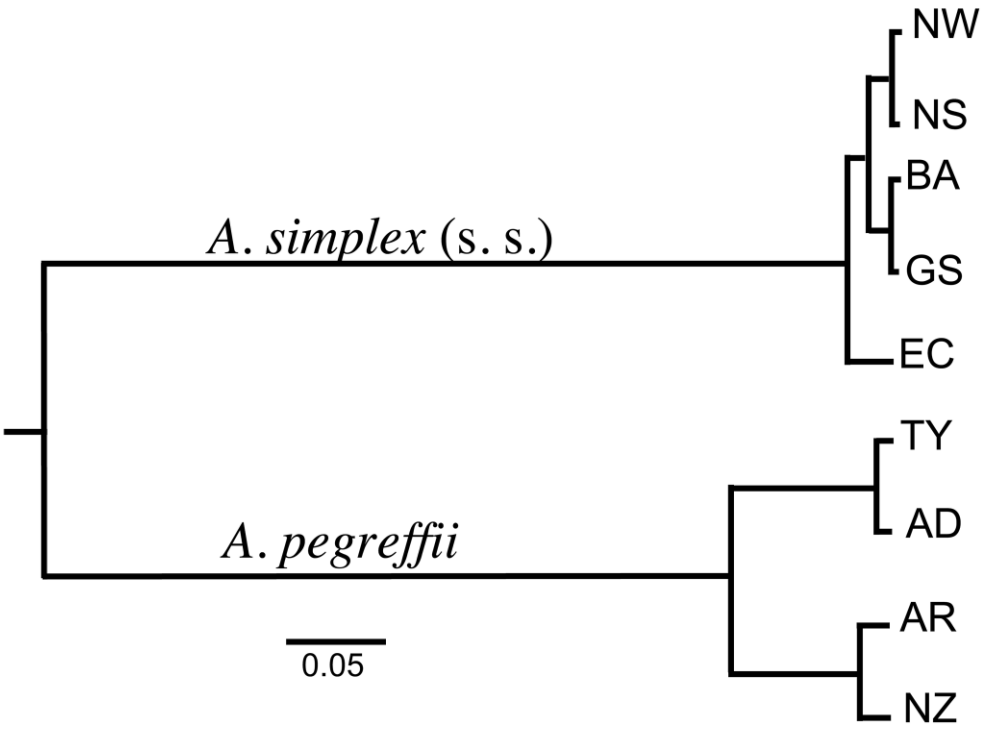

Fig. 4. The unweighted pair group method of analysis (UPGMA) cluster based on Nei's genetic distance values inferred from allelic frequencies calculated at five microsatellite loci (i.e. *Anisl 10535*, *Anisl 05784*, *Anisl 00875, Anisl 07132, Anisl 08059* ~~and *Anisl 7*~~), showing the genetic relationship between populations and species here studied (i.e . *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; AR: Argentine coast.

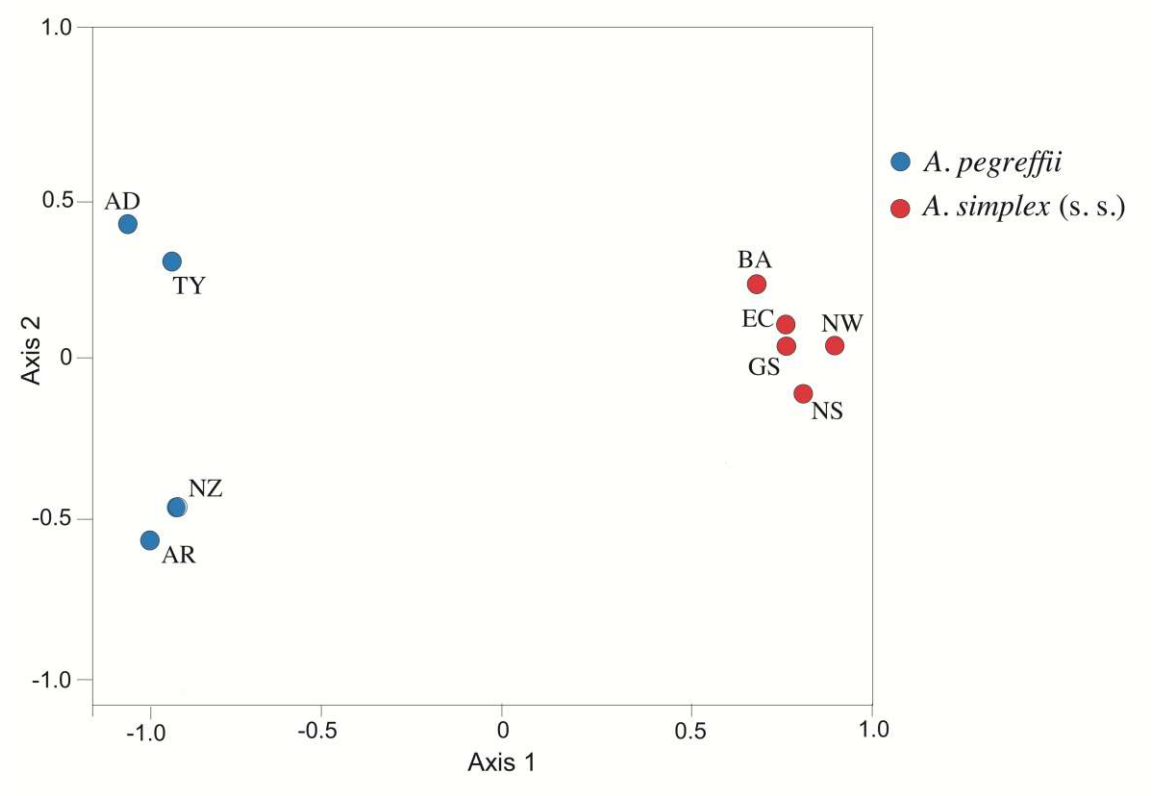

Fig. 5. Non-metric multidimensional scaling (nMDS) ordination plot inferred from Euclidean distance values obtained from the allele frequencies observed at five microsatellite loci (i.e. *Anisl 10535*, *Anisl 05784*, *Anisl 00875, Anisl 07132, Anisl 08059* ~~and *Anisl 7*~~), showing the genetic relationships 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; AR: Argentine coast.