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**SPATIAL AND TEMPORAL VARIATION IN POPULATION GENETIC STRUCTURE
OF ANISAKID NEMATODES**

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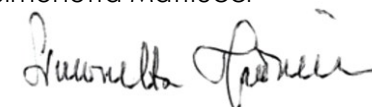
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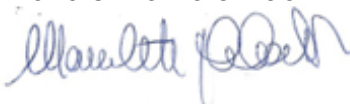
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1. INTRODUCTION

1.1 Basic biology of anisakid nematodes

The genera *Anisakis*, Dujardin, 1845, *Phocanema* (Myers, 1959) and *Contracaecum* Railliet and Henry, 1913, include nematode species which are parasites of the alimentary tract of marine mammals. These marine parasites display an indirect life cycle that relies on various hosts within marine food webs. With reference, from this point onward, to the species of the genus *Contracaecum* that mature in pinnipeds, adult nematodes of the genera *Anisakis*, *Phocanema* and *Contracaecum* colonize the digestive systems of marine mammals (cetaceans and pinnipeds) which act as definitive hosts. In these hosts, adults reproduce and release eggs into the marine environment via feces. Once hatched, free-swimming larvae emerge and undergo developmental moults before being consumed by small crustaceans, which serve as first intermediate hosts. The larvae reach the third stage (L3) within crustacean hosts and subsequently move up the food web being predated by paratenic hosts represented by fishes and cephalopods. In these intermediate hosts, the larvae accumulate as predation continues along the trophic chain, leading to significant larval concentration in predatory fishes (Munster *et al.*, 2015; Zuo *et al.*, 2016). Once the intermediate host is ingested by a definitive host, the larvae moult to the fourth stage (L4) and develop into sexually mature adults. However, the behaviour of larvae in the fish host may be different, because in some cases larvae remain confined to the visceral cavity, while in others may migrate to the musculature, leading to a risk for humans who consume undercooked or raw fish (Cipriani *et al.*, 2018, 2022, 2024; Levsen *et al.*, 2018). Human infection, known as “anisakiasis”, is a zoonotic disease caused by the accidental ingestion of *Anisakis* larvae, and it represents a global emerging issue due to the growing popularity of traditional raw and slightly processed fish dishes (Bao *et al.*, 2017; Mehrdana and Buchmann, 2017; Guardone *et al.*, 2018; Mattiucci *et al.*, 2017). Among the ten species so far genetically characterized in the genus *Anisakis*, *A. simplex* (s. s.) and *A. pegreffii* are reported as the most significant causative agents in many cases of human anisakiasis (Mattiucci *et al.*, 2011, 2013, 2017; Arai *et al.*, 2014; Lim *et al.*, 2015; Mladineo *et al.*, 2016). However, certain *Pseudoterranova* species have also been linked to human infections (Na *et al.*, 2013; Cavallero *et al.*, 2016; Arizono *et al.*), and some *Contracaecum* species are considered potentially infective (Shamsi and Butcher, 2011). Moreover, there is an increasing scientific interest on the impact of climate change and ecosystem alterations on the ecology and epidemiology of anisakids. These changes could potentially exacerbate risks for human health and impact parasite’s behaviour in their definitive and intermediate hosts (Jenkins *et al.*, 2013; Zarlenga *et al.*, 2014).

The following section provides an overview of the current taxonomy of anisakid species within the genera *Anisakis*, *Phocanema*, and *Contracaecum* (focusing specifically on species that mature in pinnipeds) that have been genetically identified to date (see Mattiucci *et al.* 2018, for a more exhaustive review).

1.2 Genetic approach in the study of anisakids nematodes

The application of molecular genetic methodologies has significantly advanced the taxonomy of anisakid nematodes over the past 25 years. Historically, these parasite species were mostly described using the "morphological species concept," which focused on visible structural features. However, genetic speciation often does not correlate with morphological differences, suggesting that the actual number of biological species likely exceeds the current number of nominal species, which are predominantly identified based on morphology. This discrepancy has led to the adoption of Mayr's "Biological Species Concept" (Mayr, 1963) in anisakid taxonomy, which defines species based on reproductive isolation. Advances in molecular techniques have demonstrated that morphological traits alone are insufficient for distinguishing "true" species within genera such as *Anisakis*, *Pseudoterranova*, and *Contracaecum* (Mattiucci and Nascetti, 2008). Over the last two decades, the use of genetic markers has revealed numerous sibling species with reproductively isolated gene pools, despite their morphological similarity, aligning with the concept of "biological species": multilocus allozyme electrophoresis (MAE), sequence analysis of the mitochondrial cytochrome oxidase 2 (mtDNA *cox2*) ((Valentini *et al.*, 2006); Mattiucci *et al.*, 2014), the ITS region of rDNA (Nadler *et al.* 2005), and the small subunit of rRNA (*rrnS*) genes (Nadler *et al.* 2005; Mattiucci *et al.* 2014); direct sequencing of nuclear DNA, such as the elongation factor - 1 alpha 1 of nuclear DNA (*EF1 α -1*) (Mattiucci *et al.* 2016) and the metalloproteinase 10 (*Nas-10*) nuclear DNA (Palomba *et al.*, 2020); the DNA microsatellite loci (Mattiucci *et al.* 2019).

1.2.1 Multilocus allozyme electrophoresis (MAE)

The introduction of multilocus allozyme electrophoresis (MAE) has been particularly influential in supporting the BSC for anisakids. Early applications of MAE analyzed 19–24 enzyme loci in large samples of *Anisakis* collected from various intermediate/paratenic, and definitive hosts. These studies demonstrated reproductive isolation and the absence of gene flow between sympatric and allopatric cryptic species, thereby establishing their specific status (rews and Chilton, 1999; Nadler and De León, 2011). Polymorphic loci deviating from Hardy–Weinberg equilibrium, as evidenced by heterozygosity deficits in sympatric populations or indirect estimates of restricted gene flow, refuted the null hypothesis of a "single panmictic species." These findings revealed substantial genetic heterogeneity within certain morphotypes of *Anisakis* and led to the discovery of cryptic species within several nominal taxa ((Mattiucci *et al.*, 1997), 2014; Nascetti *et al.*, 1993). However, MAE display several limitations. First of all, the methodology requires fresh or frozen samples, which are not always readily available, particularly for adult-stage parasites. Moreover, MAE data are not very effective for building strong phylogenetic trees for closely related groups with high genetic differences, instead, they work better with distance-based tree methods (Mattiucci and Nascetti, 2008).

1.2.2 Mitochondrial DNA loci

Among mitochondrial DNA loci, the mitochondrial cytochrome oxidase II gene (mtDNA *cox2*) is one of the most frequently used markers (Valentini *et al.*, 2006; Mattiucci *et al.*, 2008), along with the small subunit of rRNA (*rrnS*) within the mitochondrial genome (Mattiucci *et al.*, 2014; Nadler *et al.*, 2005). Additionally, primers targeting the mitochondrial cytochrome oxidase I gene (mtDNA *cox1*) have been developed (Cross *et al.*, 2007; Kijewska *et al.*, 2009). Mitochondrial genes are particularly useful due to their lack of recombination, higher mutation rates, smaller effective population sizes, and maternal inheritance. These characteristics make them valuable for both phylogenetic ((Cavallero *et al.*, 2011); Mattiucci *et al.*, 2014; Nadler *et al.*, 2005; Valentini *et al.*, 2006) and phylogeographic studies (Baldwin *et al.*, 2011; Blažeković *et al.*, 2015); Cross *et al.*, 2007; Kijewska *et al.*, 2009). In the context of its application within the *A. simplex* (s. l.) species complex, this gene has provided insights into potential population subdivisions and phylogeographic patterns, as well as on genetic variability at both interspecific and intraspecific level. Haplotype distributions of mtDNA *cox2* have been explored in both definitive and intermediate hosts of *A. pegreffii* from the Adriatic Sea (Mladineo *et al.*, 2012) and in *A. simplex* (s.s.) from *Merluccius merluccius* in northeastern Atlantic waters (Ceballos-Mendiola *et al.*, 2010). More recently, studies on mtDNA *cox2* haplotypes have revealed genetic differentiation and substructuring within populations of *Anisakis* spp., collected from different fish hosts and fishing

grounds (Baldwin *et al.*, 2011; (Klapper *et al.*, 2015). The mtDNA *cox2* locus has also shown its utility in distinguishing *A. pegreffii* and *A. berlandi* in sympatric areas of Southern ocean waters off Argentina (Irigoitia *et al.*, 2018, 2021). However, data on the population genetic structure of these species in this geographic area, based on mtDNA *cox2*, remain limited. Instead, analyses performed combining both SSRs loci and mtDNA *cox2* sequences have identified two distinct panmictic populations corresponding to *A. pegreffii* and *A. berlandi* in waters off the New Zealand coast (Bello *et al.*, 2021).

1.2.3 Direct sequencing and RFLP-PCR analysis of the nuclear ITS rDNA

Anisakid species identified through allozyme analysis have provided the foundation for DNA-based methods, such as RFLP-PCR analysis of the nuclear ITS region of rDNA (D'Amelio *et al.*, 2000) and direct sequencing of the same region (Nadler *et al.*, 2005). The internal transcribed spacer (ITS), located between the SSU and LSU rDNA regions, is divided into ITS-1 and ITS-2 by the 5.8S rDNA gene. While ITS-1 is more variable due to the presence of repeat units, ITS-2 tends to be more conserved (Van Herwerden *et al.*, 1999). Direct sequencing of the ITS region has supported phylogenetic studies, highlighting the existence of *Anisakis* species previously identified by allozymes through analyses of evolutionary lineages (Mattiucci *et al.*, 2014). RFLP patterns using restriction enzymes like *HhaI*, *HinfI*, and *TaqI* have been useful in differentiating species, producing specific diagnostic restriction patterns (D'Amelio *et al.*, 2000). However, ITS-based markers have limitations too. The ITS region is a multicopy gene subject to concerted evolution, hence its repetitiveness may violate Hardy–Weinberg assumptions (Elder, and Turner, 1995; Ganley and Kobayashi, 2007). In addition, it has been suggested that ITS-based marker alone may fail to distinguish pure specimens of *A. pegreffii* and *A. simplex* (s.s.) or detect hybrids, suggesting that a multimarker approach is recommended for their accurate identification, especially in recognition of hybrid categories (Mattiucci *et al.*, 2016).

1.2.4 Direct sequencing of the EF1 α -1 nDNA and the Nas10 nDNA

Recent advances in molecular techniques have provided new nuclear markers based on SNPs identification for distinguishing *Anisakis* species, though the exploration of such markers, giving the current state of the art, remains limited. One significant development involves the elongation factor EF1 α -1 nDNA gene. Mattiucci *et al.* (2016) designed primers for sequencing this gene in *A. pegreffii* and *A. simplex* (s.s.), previously identified through allozyme analysis. A 409-bp fragment of the EF1 α -1 nDNA region revealed two fixed diagnostic nucleotide positions. These fixed nucleotide sites were validated on 689 specimens from both sympatric and allopatric populations, confirming their reliability as markers for species identification.

Another notable nuclear marker recently developed is the metalloproteinase-10 (*Nas10*) gene, a 451-bp locus sequenced in 219 specimens of the *A. simplex* (s. s.) complex. This marker displayed fixed, species-specific nucleotide positions, achieving 100% diagnostic accuracy for species determination. Additionally, primers were developed for amplification-refractory mutation system (ARMS) PCR, enabling efficient genotyping through a tetra-primer assay (Palomba *et al.*, 2020).

1.2.5 Genotyping of nuclear microsatellites (SSRs) loci

Microsatellites are codominant markers composed of repetitive DNA sequences that are widely distributed across chromosomes (Avice, 1994). Due to their high mutation rates, microsatellites are especially useful for examining detailed genetic population structures. Mladineo *et al.* (2017a) developed a set of microsatellite markers for *A. simplex* (s.s.) and *A. pegreffii*. Although these markers were highly variable, they did not clearly distinguish between the two species, as the analysis included only three *A. simplex* (s.s.) specimens and a single *A. pegreffii* population from the Adriatic Sea. Further work led to the creation of a set of seven microsatellite loci, which were later tested on a large number of specimens encompassing the *A. simplex* (s. s.) species complex (Mattiucci *et al.*, 2019; Bello *et al.*, 2020, 2021). The high level of polymorphism displayed by these markers has made them valuable tools for studying genetic variations within populations. The extensive sampling of these species from both sympatric and allopatric areas has enabled gene flow between distant populations and calculate genetic differentiation and variability within populations. This approach, combined with other genetic-molecular techniques, offers a powerful multi-marker method to address questions related to their taxonomic status, but also to provide data on population genetics, molecular ecology, and epidemiology of these parasitic nematodes.

1.3 Current taxonomy of nematodes of genus *Anisakis*

To date, ten species have been described and classified within the genus *Anisakis*: *A. simplex* (s. s.), *A. pegreffii*, *A. berlandi*, *A. typica* sp. A, *A. typica* sp. B, *A. nascettii*, *A. ziphidarum*, *A. physeteris*, *A. paggiae*, and *A. brevispiculata*. These species are distinguished by diagnostic genetic markers, possess distinct gene pools, and are reproductively isolated (Cipriani *et al.*, 2022); Mattiucci *et al.*, 2018). Concatenated phylogenetic analysis of nuclear and mitochondrial genes have identified four distinct clades within this genus of marine endoparasites (Valentini *et al.*, 2006; Mattiucci and Nascetti, 2008; Mattiucci *et al.*, 2018).

1.3.1 Ecological aspects of the *Anisakis simplex* (s. l.) complex

For the purposes of this thesis, the focus will primarily be on Clade I. This clade consists of the *A. simplex* (s. l.) complex, which includes *A. berlandi*, *A. pegreffii*, and *A. simplex* (s. s.). These parasitic nematodes preferentially infect marine mammals from the Superfamily Delphinoidea (such as Delphinidae, Phocoenidae, and Monodontidae). As adults, they exhibit shared morphological traits: (i) a ventriculus that is longer than wide and often sigmoid in shape, and (ii) long, thin, and unequal male spicules (Mattiucci *et al.*, 2014).

The distribution of *A. simplex* (s.s.) ranges from approximately 35°N to the circumpolar Arctic Seas and, additionally, this species has been identified along the Pacific coasts (as reviewed by Mattiucci *et al.*, 2018). In these areas, *A. simplex* (s.s.) has been documented in over 50 species of pelagic, benthopelagic, and demersal teleost fish, as well as in four squid species (Mattiucci *et al.*, 2018). In contrast, *A. simplex* (s.s.) is less frequently encountered in southern waters. Larval stages of the species have been observed in the Alboran Sea and infrequently in the Mediterranean, possibly due to the migratory patterns of its paratenic hosts such as *Merluccius merluccius*, *Scomber scombrus*, and *Thunnus thynnus* (Levsen *et al.*, 2017; Mladineo *et al.*, 2017), and definitive hosts, specifically *Tursiops truncatus* and *Stenella coeruleoalba* (Blažeković *et al.*, 2015; Mladineo *et al.*, 2017a).

On the contrary, *A. pegreffii* shows a more widespread distribution. It represents the dominant species of *Anisakis* in the Mediterranean Sea, being present in many pelagic and demersal fish species (see Palomba *et al.*, 2023 and references therein). Its northern limit is represented by the Iberian Atlantic Coast, around the Biscay Bay, where it is identified frequently in sympatry with the sibling species *A. simplex* (s. s.) (Abollo *et al.*, 2001; Cipriani *et al.*, 2015; Marques *et al.*, 2006; Mattiucci *et al.*, 2004, 2007; Pontes *et al.*, 2008). *A. pegreffii* appears only sporadically in northern waters of the northeast Atlantic Ocean. Its presence has been recorded in the species *Scomber scombrus* collected from the southern Norwegian Sea, probably linked to changes in the

migratory behaviour of southern *S. scombrus* stocks in these waters (Levsen *et al.*, 2017a, b). Indeed, in the Southern Hemisphere, *A. pegreffii* ranges between latitudes of 30°S and 60°S, where it has been found in both larval and adult stages (Mattiucci *et al.*, 2014, 2017), with high abundances of adults recorded in dolphins, primarily within the Delphinoidea family, as well as off in Southern African coast (see Mattiucci *et al.*, 2018).

Anisakis berlandi shows a discontinuous distribution across the Austral region: its presence has been recorded, at the adult stage, in several cetacean species from the Pacific Chilean waters, the South Shetland Islands, the New Zealand waters and the South African Atlantic Coast (Klimpel *et al.*, 2010; Mattiucci *et al.*, 2014; Mattiucci *et al.*, 2018). Definitive hosts include *Globicephala melas* and *Grampus griseus* in which the species has been recorded in syntopy with *A. pegreffii* in sympatric areas from off New Zealand coast, plus South African and Chilean Pacific waters (Mattiucci *et al.*, 2018). Less frequently the species has been identified at the adult stage in *Kogia sima*, *Mirounga leonina* and *Ziphius cavirostris* (Mattiucci *et al.*, 2017; Mattiucci and Nascetti, 2008). As regarded the paratenic hosts, larvae of *A. berlandi* has been recorded in several fish species from New Zealand waters (Mattiucci *et al.*, 2014; Bello *et al.*, 2020), as well as from off Southern Chilean (Mattiucci *et al.*, 2018) and Argentine coasts (Irigoitia *et al.*, 2018, 2021).

1.4 Current taxonomy of the genus *Phocanema*

The taxonomy of genus *Phocanema* has been investigated by allozyme markers (Paggi *et al.*, 1991) and phylogenetic analysis of the nuclear LSU rDNA gene, the ITS rDNA region and the mtDNA *cox2* gene (Bao *et al.*, 2022 and references therein). Currently, it includes *Pseudoterranova kogiae*, *P. ceticola*, and the sibling species complex *Phocanema decipiens* (s. l.), which encompasses *P. decipiens* (s. s.), *P. cattani*, *P. krabbei*, *P. bulbosa*, *P. azarasi*, and *P. decipiens* E (Paggi *et al.*, 2001; Mattiucci & Nascetti, 2008; Timi *et al.*, 2014). Recently, the resurrection of the genus *Phocanema* has been proposed for this complex (see Bao *et al.*, 2023), while *P. kogiae* and *P. ceticola* remain within the genus *Pseudoterranova*. The *P. decipiens* (s. l.) complex parasitizes pinnipeds in its adult stage, whereas *P. kogiae* and *P. ceticola* have cetaceans in the family Kogiidae as their definitive hosts (Cipriani *et al.*, 2024).

1.4.1 Ecological aspects of the *Phocanema decipiens* (s. l.) species complex

The following section provides an overview of the species within the *P. decipiens* (s. l.) complex (see Mattiucci and Nascetti, 2008, for further details). These species are selected based on their distinct geographic distribution and host preferences, which are relevant to the focus of this thesis.

P. decipiens (s. s.), formally described by Paggi *et al.* (2000) based on diagnostic morphological traits, displays a wide distribution Arctic and sub-Arctic regions, including the North-East Atlantic (e.g., Scottish, Icelandic, and Norwegian waters), Canadian Atlantic, and Pacific waters. At the adult stage, it parasitizes *Phoca vitulina* and *Halichoerus grypus* in the Atlantic and *Zalophus californianus* in the Pacific, while larvae have been found in gadoid fishes (Brattey and Stenson, 1993; Paggi *et al.*, 1991; Mattiucci *et al.*, 1998; Nadler *et al.*, 2005). Sympatric infections with other species, such as *P. krabbei* and *P. bulbosa*, occur in these regions (Paggi *et al.*, 2001).

P. krabbei has been detected in North-East Atlantic waters, parasitizing *Halichoerus grypus* and *Phoca vitulina*, often in mixed infections with *P. decipiens* (s.s.). Morphologically distinct by shorter spicules and caudal papilla arrangement, at the L3 stage it infects *Gadus morhua* and other gadoids (Paggi *et al.*, 1991).

P. bulbosa is found in the Barents and Norwegian Seas, Canadian Atlantic, and the Sea of Japan. Its definitive host is *Erignathus barbatus*, in which it's often detected in mixed infections with *P. azarasi* (Mattiucci *et al.*, 1998), while it uses benthic flatfishes as intermediate hosts (Paggi *et al.*, 1991).

1.5 Current taxonomy of the *Contracaecum* species

The genus *Contracaecum*, originally defined by Railliet and Henry (1912), encompasses approximately 50 species, primarily parasitizing pinnipeds and fish-eating birds. Several studies have identified two distinct evolutionary clades within this genus, differentiated by their definitive host preferences. Molecular and allozyme clustering analyses have shown that *Contracaecum* species parasitizing pinnipeds are genetically more closely related to species of the genus *Phocascaris* (*P. phocae* and *P. cystophorae*) than to other *Contracaecum* species from fish-eating birds (Nascetti *et al.*, 1990). This clade's monophyly is supported by nuclear rDNA and mitochondrial DNA (mtDNA *cox2*) phylogenetic analyses (Nadler *et al.*, 2000; Mattiucci *et al.*, 2008). Here we are referring to the species of the genus *Contracaecum* maturing in pinnipeds. The *Contracaecum osculatum* (s.l.) is a complex of sibling species, and it will be examined in the following paragraphs due to its relevance in the context of this thesis.

1.5.1 Ecological aspects of the *Contracaecum osculatum* (s. l.) species complex

To date, five members of the *C. osculatum* (s. l.) complex have been genetically first discovered by allozyme markers, demonstrating their reproductive isolation. These include three Arctic sibling species, i.e. *C. osculatum* sp. A, *C. osculatum* sp. B, and *C. osculatum* (s. s.) as described in Nascetti *et al.* (1993), and two Antarctic species, i.e. *C. osculatum* sp. D and *C. osculatum* sp. E (see Orecchia *et al.*, 1994). These species display distinct geographical distribution and a marked host specificity.

The species *C. osculatum* A has been documented in the Norwegian and Barents Seas, in the Canadian Atlantic and Pacific, in Icelandic waters, and in the Sea of Japan, spanning latitudes from 40°N to 80°N. Its main definitive host is the bearded seal *Erignathus barbatus*, though it has also been found in the grey seal *Halichoerus grypus* in the North Atlantic (Bratney and Stenson, 1993; Nascetti *et al.*, 1993). It has been genetically identified also in the Steller sea lion *Eumetopias jubatus* in the Sea of Japan (Mattiucci *et al.*, 1998; Paggi *et al.*, 1998), with larvae observed in the gadoid fish *Theragra chalcogramma* from the same region (Mattiucci *et al.*, 1998).

Spanning lower Arctic latitudes with respect to its congeneric species above, *C. osculatum* B has been identified in a broader variety of definitive hosts. Firstly genetically detected in the harp seal *Pagophilus groenlandicus*, in the harbour seal *Phoca vitulina*, and in the grey seal *Halichoerus grypus* in the North Atlantic (Bratney and Stenson, 1993; Nascetti *et al.*, 1993), *C. osculatum* B was later found in harbour seals and California sea lions *Zalophus californianus* in the North Pacific (Mattiucci *et al.*, 1998).

As regarding *C. osculatum* (s. s.), referred to as *C. osculatum* C by Nascetti *et al.* (1993), it was genetically characterized from the grey seal *Halichoerus grypus*, in which it has been found in syntopy with *C. osculatum* sp. B in sympatric areas from Icelandic waters (Nascetti *et al.*, 1993). Moreover, *C. osculatum* (s. s.) is the only species in the *C. osculatum* complex found in the Baltic Sea and has not been reported from the western Atlantic or Pacific regions.

The Antarctic species *C. osculatum* sp. D and *C. osculatum* sp. E were identified by Orecchia *et al.* (1994) using allozyme markers. These two species coexist in the Weddell seal *Leptonychotes weddellii* and have been documented in both the Weddell and Ross Seas. Larval stages have been identified in various fish families, including Channichthyidae, Bathydraconidae, and Nototheniidae, with a differential distribution among these hosts (see Mattiucci and Nascetti, 2007).

1.6 Anisakids as ecological indicators

Marine parasites have increasingly been recognized as valuable indicators in biological and ecological assessments of marine ecosystems (Palomba *et al.*, 2023). They serve as proxies for various ecological phenomena, including the structure of food chains (Thompson *et al.*, 2005), pollution levels (Sures, 2004), and the effects of global climate change (Brooks and Hoberg, 2007; Poulin, 2006). Additionally, they are utilized in evaluating anthropogenic impacts and environmental stressors, and overall ecosystem health (Marcogliese, 2005). Anisakid parasites, as integral components of aquatic ecosystems, not only contribute to population dynamics and community structure but also provide insights into the biodiversity of marine habitats.

The transmission dynamics of anisakid nematodes are closely linked with the trophic relationships among their hosts, thus they are embedded in food webs. Consequently, the completion of their complex life cycles is dependent on the stability of these trophic interactions. Anthropogenic disturbances to marine ecosystems which alter host population size, such as pollution, by-catch of marine mammals and overfishing, viral diseases, and the fragmentation of marine habitats, could significantly impact the genetic and demographic viability of anisakid parasites populations. Lower host populations sizes and reduced migration rates in fragmented populations may lead to reduced anisakid parasite demography, making them more vulnerable to the effects of genetic drift (Mattiucci and Nascetti, 2008). The latter in turn manifests as a decrease in genetic variability within parasites gene pools, being characterized by a loss of allelic diversity, reduced heterozygosity, and lower polymorphism rates.

The extent to which a species is affected by such disturbances is influenced by its specialization and dispersal capacity. Species exhibiting a high dispersal capability have higher possibilities to face anthropogenic impacts. For instance, they are more resilient to habitat loss and fragmentation, because of their larger range of distribution and multiple populations act as potential sources of diversity (Jones *et al.*, 2007). In anisakid nematodes, gene flow is largely determined by the movement of their host species (Mattiucci *et al.*, 1997, 2008, 2018), and their abundance, again, is correlate with host density (Mattiucci *et al.*, 2015). Consequently, variations in host density and its dispersal patterns are likely to affect the genetic diversity distribution among anisakid parasite populations (Mattiucci and Nascetti, 2007; Zarlenga *et al.*, 2014).

To date, it has been recognized that two primary strategies can be employed to evaluate the genetic impacts of habitat disturbances on anisakid populations (Mattiucci *et al.*, 2015). They are the comparative analysis of genetic variability across populations inhabiting ecosystems with varying levels of disturbance (spatial dimension), and the analysis of genetic variability within populations from the same geographical region across timescales (temporal dimension). As regarding the first

point, studies on genetic variability and infection levels in anisakid populations across geographically distinct regions of the Boreal and Austral hemispheres, exhibiting differing degrees of habitat disturbance, have been carried out. Mattiucci and Nascetti (2007) reported significantly higher genetic variability in anisakid populations from the Austral regions compared to those from the Boreal regions, with heterozygosity values at the MAE analysis in the Austral populations being approximately twice that of the Boreal populations. The elevated genetic diversity in Antarctic populations has been attributed to lower levels of habitat disturbance. In this regions, in fact, impacts from overfishing, by-catch, pollution, and climate change are minimal. Such conditions allow host species to maintain larger population sizes, supporting greater anisakid parasite populations and minimizing the effect of genetic drift. Moreover, higher infection rates have been observed in Antarctic hosts (Mattiucci and Nascetti, 2008, and references therein), which further corroborate this hypothesis when compared to those observed in Arctic hosts. These studies bring to light that the high genetic variability and parasitic burdane in Antarctic anisakid populations are consistent with the stability and integrity of the local food webs. These optimal conditions facilitate the completion of anisakid life cycles, reflecting the health of this ecosystem.

Monitoring genetic variability using molecular markers could offer valuable insights into trophic web stability and marine biodiversity. Future studies should focus on the exploration of the temporal and spatial aspect of genetic monitoring in anisakid populations, as this is essential for assessing ecosystem health and stability in response to environmental and anthropogenic changes.

1.7 Aims of the study

The conservation of marine biodiversity and the stability of trophic webs are increasingly under threat from a variety of environmental and anthropogenic disturbances, including overfishing, pollution, and global climate change. Innovative approaches are requested to monitor the impacts on biodiversity at both species and genetic level caused by these stressors. Anisakid nematodes provide a valuable tool for studying ecosystem dynamics, as their life cycles are deeply embedded within trophic webs, and their genetic variability often reflects the health and stability of their host populations.

This research aims to use these features of marine anisakid parasites to explore key issues in marine biodiversity and ecosystem health. Particular attention will be given to certain species belonging to the genera discussed above, *Anisakis* and *Contracaecum*, which inhabit geographical regions with differing ecological conditions. The Arctic-Boreal and Antarctic regions, characterized by different levels of anthropogenic impact, provide an ideal opportunity to explore how human and environmental pressures affect genetic variability of parasite populations.

The specific aims of this work are:

1. To define biodiversity at the species and gene levels by analysing the genetic variability of anisakid populations across spatial and temporal scales. This objective involves a comparative study of metapopulations of the species *Contracaecum osculatum* B, a heteroxenous parasite infecting fishes and pinnipeds in the Arctic regions. The choice of *C. osculatum* B as a “model species” is based on its suitability for investigating trophic web stability across a temporal scale. The life-cycle of the species, which takes place in Arctic and Antarctic regions, involves fishes as intermediate/paratenic hosts and pinnipeds as definitive hosts. Historical samples preserved for over 35 years have provided a rare opportunity to estimate changes in genetic variability over time in the Arctic region, an area increasingly affected by environmental and anthropogenic pressures. The significant innovation in this study has been provided by the development of novel microsatellite markers in the species *C. osculatum* B, which have proven to be applicable to the whole sibling species complex, both the Arctic and in the Antarctic ones. These markers offer an assessment of the genetic variability, not only across geographically distinct regions but also over such broad temporal scale. The comparison of historical and current populations may allow to understand of how genetic variability may be affected by increased levels of habitat disturbance. This will be the object of **Paper no. 1**.

2. The second objective focuses on the study of admixture between the species *Anisakis pegreffii* and *Anisakis simplex* (s.s.), parasites of fishes, squids and cetaceans, in their sympatric area of

North-East Atlantic waters. Their distribution in European waters is shaped by both biotic and abiotic factors, and both species co-occur in sympatry along the Atlantic coasts of Spain. To address this objective, this research has employed a multilocus genetic approach, combining molecular markers already broadly tested in the *A. simplex* (s. l.) species complex and discussed above, with newly developed nuclear markers based on SNP detection in sequenced superoxide dismutase (*Sod* nDNA) and adenylate kinase (*Adk* nDNA) genes. This comprehensive framework allows for a detailed analysis of genetic variability within and between *A. pegreffii* and *A. simplex* (s. s.) populations. This will be the object of **Paper no. 2**.

2. I Population genetic structure of the anisakid nematode *Contracaecum osculatum* sp. B over a temporal scale: a tool for monitoring trophic-web dynamics in Arctic Sea

Beatrice Belli, Marialetizia Palomba, Paolo Cipriani, Roberta Andolfi, Lucilla Giulietti, Miguel Bao, Giuseppe Nascetti, Simonetta Mattiucci

Abstract

Global changes in the marine Arctic ecosystem affecting the demography of fish and seal populations may alter anisakid population sizes, potentially leading to genetic erosion in parasite gene pools. *Contracaecum osculatum* sp. B is a sibling species within the *C. osculatum* (s.l.) complex, parasitizing Arctic and sub-Arctic seals and fish. This study aimed to compare parasite genetic diversity across the Nordic Seas over a 35-year time scale. Historical specimens (1985–1986) from seals were compared with contemporary specimens (2021–2022) from fish in the same regions. Parasites were first identified through sequence analysis of mtDNA *cox2* and ITS rDNA, followed by genotyping at seven newly developed SSRs nDNA loci. The population genetic structure of *C. osculatum* sp. B revealed the existence of two parasite subpopulations. This finding appears to be mostly influenced by the population structure of the seal hosts in the study area. High genetic variability was observed at both nuclear and mitochondrial levels. In the gene pool of the contemporary parasite population, some rare haplotypes in mtDNA *cox2* and rare alleles at SSRs-DNA loci have been lost compared to the historical population, while novel unique alleles and haplotypes have simultaneously emerged. Overall, these findings seem suggest the occurrence of an initial decline in parasite population size, followed by a period of demographic stability and, more recently, a population increase. Similar demographic patterns have been documented in the host populations over the same time scale. Genetic polymorphisms in anisakids across temporal scales may help unravel and monitor trophic web dynamics in the Arctic marine ecosystem under global change.

Key words: *Contracaecum osculatum* sp. B, fish, seals, microsatellite DNA loci, mtDNA *cox2*, genetic structure, genetic variability, trophic-web

2.1 Introduction

Trophically transmitted marine helminths, which have heteroxenous life cycles with free-living stages and multiple hosts across different trophic levels, are recognized as ecological indicators of food web interactions and ecosystem health (Lafferty et al., 2008a; Mattiucci and Nascetti, 2008; Mattiucci et al., 2015; Palomba et al., 2023). Being closely linked to trophic transmission and host specificity, these helminth endoparasites require stable trophic webs, and diverse and abundant definitive and intermediate host populations to complete their life cycle (Marcogliese, 2005; Mattiucci and Nascetti, 2008; Palomba et al., 2023; Wood and Vanhove, 2022). Any anthropogenic stressor that causes a decline in host populations may also affect the population size of their endoparasites over time (Marcogliese, 2005; Nichols and Gómez, 2011; Palomba et al., 2023), and thus parasite abundance can be expected to fluctuate in response to global change effects (Mattiucci and Nascetti, 2008; Altman and Byers, 2014; Wood et al., 2023).

Among these stressors, rising ocean temperatures associated with climate change may alter host population abundance, distribution, and migratory routes, consequently affecting parasite transmission and distribution (King et al., 2015; Levsen et al., 2018, 2022; Palomba et al., 2023; Diez et al., 2024; Mattiucci et al., 2025). Indeed, rising ocean temperature may also directly affect the free-living stages of parasites, impacting their survival, development rates, and dispersal, altering population size and distribution patterns. Other anthropogenic effects, such as pollution, may weaken host immune defences against specific parasite species, leading to increased transmission, or may have the opposite effect by causing direct mortality of free-living infectious stages (Sures and Nachev, 2022). Over-exploitation of natural resources, such as overfishing, may reduce host density, thereby decreasing parasite transmission and abundance (Dobson and May, 1987; Lafferty et al., 2008b). Finally, both natural (e.g. virus spread) and anthropogenic impacts (e.g. hunting, bycatch, ship collisions, noise disturbance, pollution) affecting populations of definitive hosts of these parasites, such as marine mammals (e.g. cetaceans and pinnipeds), may reduce their population size (Härkönen et al., 2006; Blanchet et al., 2021; Silva et al., 2021), thereby decreasing parasite recruitment. Overall, these phenomena impact parasite genetic polymorphism and variability (Mattiucci and Nascetti, 2008), potentially leading to a loss of genetic diversity and polymorphism in the gene pool of a specific parasite population due to genetic drift (Bullini et al., 1986; Mattiucci and Nascetti, 2008).

The Arctic marine ecosystem represents a valuable model for wildlife ecology studies, allowing for comparative analysis with other oceanographic areas. Arctic parasites, like their hosts, face new challenges as climate change alters host-parasite interactions (Davidson et al., 2011). In this context, anisakid nematodes have been proposed as indirect indicators of trophic web stability in

marine ecosystems across spatial and temporal scales, based on measurable features such as genetic variability and parasitic load estimates (Mattiucci and Nascetti, 2008; Mattiucci et al., 2015; Palomba et al., 2023). However, identifying long-term trends in marine endoparasites remains challenging due to incomplete timeline data, variability in study methodologies, and inconsistencies in temporal genetic analyses.

In Arctic and sub-Arctic waters, three sibling species of the *C. osculatum* (s.l.) complex—*C. osculatum* sp. A, *C. osculatum* sp. B, and *C. osculatum* (s.s.)—occur in seals and fish species. These species are reproductively isolated and exhibit differential host preferences (Nascetti et al., 1993). The existence of these parasite species, initially discovered using allozyme markers (Nascetti et al., 1993), was later confirmed through sequence analysis of the ITS region of rDNA (Zhu et al., 2000) and the mitochondrial cytochrome C oxidase subunit II (mtDNA *cox2*) gene locus (Mattiucci et al., 2008). In particular, *C. osculatum* sp. B, found in the North East and North West Arctic and sub-Arctic waters, has a life cycle that primarily involves the harp seal (*Pagophilus groenlandicus*) and the common seal (*Phoca vitulina*) as definitive hosts, and to a lesser extent, the grey seal (*Halichoerus grypus*) (Mattiucci and Nascetti, 2008). Fish species, such as capelin and Arctic cod, serve as intermediate or paratenic hosts (Levsen et al., 2016, 2022).

In the present study, the genetic variation of the parasite *C. osculatum* sp. B was analysed in Arctic Norwegian waters across seal and fish hosts and over an extended timescale with the following aims: i) to assess the population genetic structure of *C. osculatum* sp. B using nuclear newly developed DNA microsatellite loci and mitochondrial (mtDNA *cox2*) loci, based on historical (1985–1986) and contemporary (2021–2022) specimens, and ii) to compare genetic variability estimates over a temporal scale, using both mitochondrial and nuclear genetic datasets. This approach aimed to explore potential relationships between parasite genetic variability and population size fluctuations over time, possibly resulting from global changes in Arctic Sea waters.

2.2 Materials and methods

2.2.1 Nematodes sampling

A total of $N = 602$ anisakid specimens of *Contracaecum osculatum* (s.l.) were genetically analyzed. This included $N = 388$ nematodes obtained from the historical repository collection of S. Mattiucci at the Section of Parasitology, Department of Public Health and Infectious Diseases, Sapienza University of Rome. These adult specimens were originally collected from several harp seal (*Pagophilus groenlandicus*) individuals and a single harbour seal (*Phoca vitulina*) from the Nordic Seas during 1985–1986 (Table 1; Fig. 1). Adult *Contracaecum* specimens were first assigned as male and female based on the morphological characters described by Fagerholm (1989). The anisakid nematodes in the contemporary sample ($N = 214$) were collected at the L3 larval stage from $N = 35$ Arctic cod (*Gadus morhua*) and pollock (*Pollachius virens*) fished in Norwegian Arctic and sub-Arctic regions between 2021 and 2022 (Table 1; Fig. 1).

Table 1. Number of specimens of *C. osculatum* (s. l.) scored in the present study at different mitochondrial and nuclear gene loci, reported with their host species, life-history stage and sampling areas. N_H, number of individual hosts examined; N_P, total number of parasites tested; N_A, number of adult nematodes; N_{L3}, number of specimens at third larval stage.

Sampling period	Area	Code area	Host species	N _H	N _P	N _A	N _{L3}	N <i>cox2</i> mtDNA	N ITS rDNA	7 SSRs DNA loci
1985-86	Barents Sea (69°22'N-39°30'E)	BS	<i>Pagophilus groenlandicus</i>	6	135	135	-	92	92	92
	Bergen (Norway)	BE	<i>Pagophilus groenlandicus</i>	5	116	116	-	24	24	24
			<i>Phoca vitulina</i>	1	16	16	-	12	12	12
	Jan Mayen (70°44'N-7°44'W)	JM	<i>Pagophilus groenlandicus</i>	3	121	121	-	80	80	80
	Tot. (1985-86)			15	388	388	-	208	208	208
2021-22	Barents Sea (75°15'N-3°17'E)	BS	<i>Gadus morhua</i>	6	60	-	60	60	60	60
	Finnmark Coast (71°13'N-24°46'E)	FI	<i>Gadus morhua</i>	12	50	-	50	50	50	50
			<i>Pollachius virens</i>	4	10	-	10	10	10	10
	Lofoten Islands (68°64'N-14°13'E)	LO	<i>Gadus morhua</i>	9	64	-	64	64	64	64
	Svalbard Islands (72°02'N-03°01'E)	SV	<i>Gadus morhua</i>	4	30	-	30	30	30	30
	Tot. (2021-22)			35	214	-	214	214	214	214
Tot.					602	388	214	422	422	422

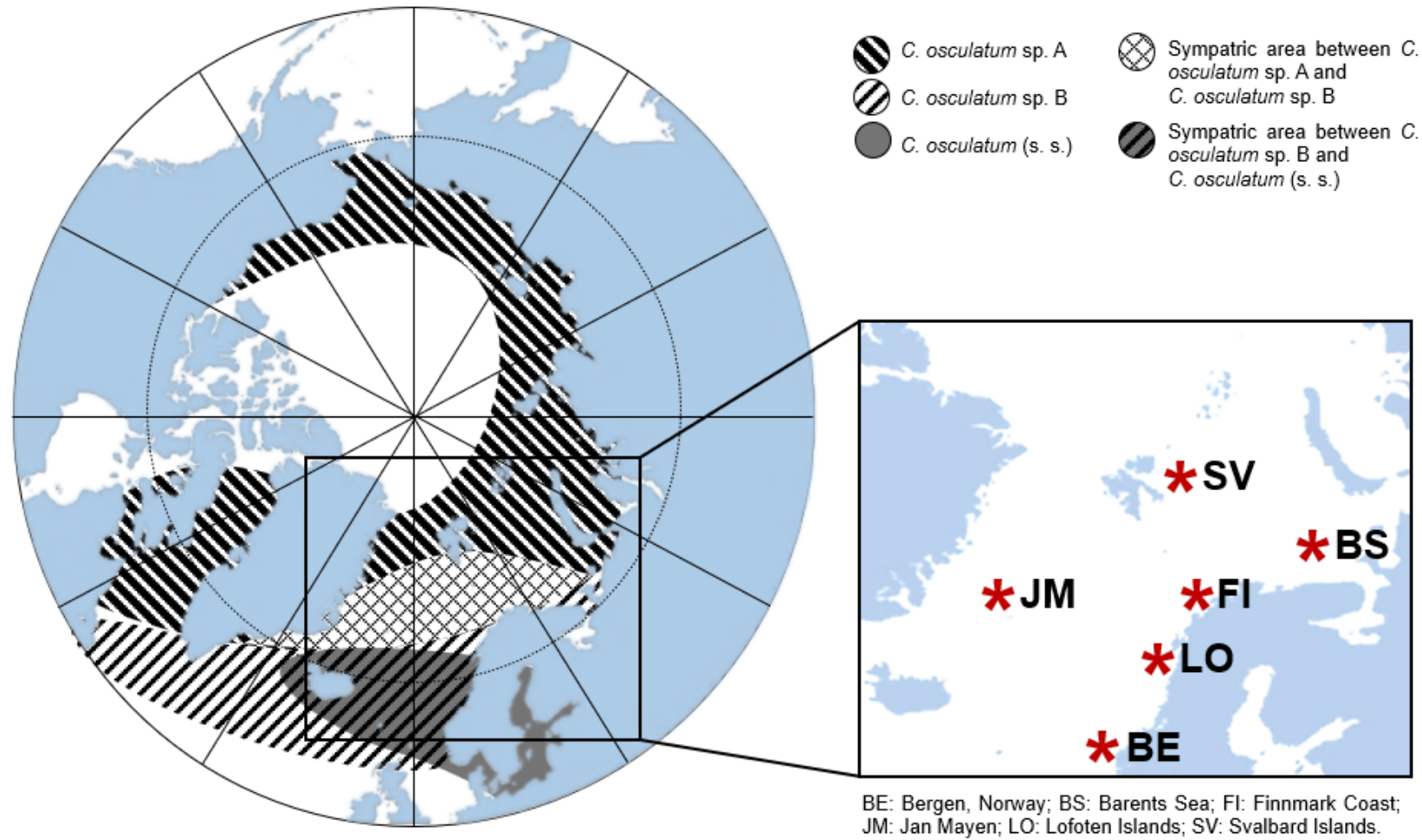


Figure 1. Collecting sites of populations of *C. osculatum* (s.l.) analysed in the present study, mapped into the geographical range of the three Arctic sibling species, i.e., *C. osculatum* sp. A, *C. osculatum* sp. B and *C. osculatum* (s. s.), as reported in Nascetti *et al.*, (1993).

2.2.2 DNA extraction

Samples underwent multiple washes in ultrapure water before DNA extraction. DNA was successfully obtained from N = 208 historical and N = 214 contemporary specimens out of the N = 602 tested. Ethanol-preserved samples were pre-treated with multiple washes in 9% Phosphate Buffered Saline (PBS) over 24–48 hours. Total DNA was extracted from a tissue portion of each larval specimen. For the adult worms, sex was determined when feasible. Only the internal body parts were used for DNA extraction, while the cephalic and caudal extremities were preserved in alcohol. DNA extraction followed the protocol recommended by the Quick-gDNA Miniprep Kit (ZYMO RESEARCH).

2.2.3 Genetic identification of *Contracaecum osculatum* (s.l.)

For genetic identification, a total of N = 422 *C. osculatum* (s.l.), including both the historical and contemporary specimens from definitive and intermediate/paratenic hosts (Table 1), were sequenced at two diagnostic genes i.e. the ITS region of rDNA (Zhu et al., 2000; Nadler et al., 2005) and the mtDNA *cox2* gene locus (Mattiucci et al., 2008). For the ITS region of rDNA, the primers NC5 (5' – GTA GGT GAA CCT GCG GAA GGA TCA TT – 3') and NC2 (5' – TTA GTT TCT TTT CCT CCG CT – 3') were used, following the PCR condition as reported in Zhu et al. (2000). The mtDNA *cox2* was amplified 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), following the PCR conditions specified in Mattiucci et al. (2008; 2015).

2.2.4 Development of DNA microsatellites (SSRs loci)

A subset of *C. osculatum* sp. B from contemporary samples, previously identified through ITS rDNA and mtDNA *cox2* sequence analyses, was used to generate a highly concentrated pooled DNA sample suitable for genome library construction and scoring of the SSRs-DNA loci. A whole-genome sequencing library was created using the Illumina TruSeq Nano DNA Library Kit and sequenced on an Illumina MiSeq platform with a Nano v2 500-cycle sequencing chip. After assembly, 99,743 merged reads were screened, of which 10,490 contained microsatellite inserts with tetra- or trinucleotide repeats of at least six units, or dinucleotide repeats of at least ten units. From these, primer design was successfully performed for 5,870 microsatellite candidates. A total of seven microsatellite loci were then selected and designated as: Co42335, Co85484, Co97601, Co121208, Co166121, Co210159, Co215165 (Table 2). Primers were designed for an optimal annealing temperature of 56°C and tested for polymorphism using 15 pooled *C. osculatum* sp. B specimens from various geographical areas. Allele sizes were determined on an ABI3730, using the GeneScan™-500 LIZ Size Standard. In the initial test for SSRs development using the subset

of *C. osculatum* sp. B samples, the number of alleles ranged from 4 to 11, ensuring a good level of polymorphism in the parasite species. Subsequently, all adult and larval *C. osculatum* sp. B specimens (reported in Table 1) were analyzed using the seven SSR markers (Table 2). Multiplex PCR amplification was optimized in a 10 µl reaction volume, containing 2–10 ng of genomic DNA, 5 µl Multiplex Master Mix (Qiagen®), double-distilled water, and 0.3 µM labeled forward and reverse primers (Table 2). The following cycling protocol was used for amplification: 35 cycles at 94°C for 30 s, 56°C for 90 s, and 72°C for 60 s. A prolonged denaturation step (95°C for 15 min) was included before the first cycle, and the final cycle was followed by a 30-minute extension at 72°C. Amplified PCR products were genotyped by an external company (Bio-Fab Research©). Electropherograms were manually checked using PeakScanner v.2 (Applied Biosystems, Waltham, MA, USA). The software MICRO-CHECKER v.2.2.3 (Van Oosterhout et al., 2004) was used to check for the presence of genotyping errors and null alleles.

Table 2. Locus name, primers sequences, number of alleles (N_A), repeat type, fragment bp size and fluorescent dye of 7 DNA microsatellite loci (SSRs-DNA) developed in the present study, in the species *C. osculatum* sp. B.

Locus name	Primer sequence 5' – 3'	N_A	Repeat type	Range size (bp)	Dye
<i>Co42335</i>	F: AAGTTTAGGGCGATTGCACG* R: AAGTGATCAACGACGAAGGC	11	(GCA) 8	65-95	ATTO565 (red)
<i>Co215165</i>	F: ACCAAGAAACGCATTCGTGC* R: ACAGCCAGCATTTGGTATCG	11	(TACT) 8	110-154	ATTO532 (green)
<i>Co97601</i>	F: GAAATAAGTCCAGGTGCACGG* R: AAATTCATCCTTTTGCGTTGAC	11	(ACA) 7	107-167	FAM (blue)
<i>Co85484</i>	F: TTATAACACGCCTTCGGAGC* R: GGGGACAAAAGCGTATCGTG	24	(TAA) 11	101-203	ATTO565 (red)
<i>Co121208</i>	F: TGAAGCAGCATCTACCATTTTCG* R: AATAGTAGCAGTGAAACACGTC	7	(TGT) 8	154-172	ATTO550 (yellow)
<i>Co210159</i>	F: ACTGTATAACGACCACATAGAGC* R: ACGTATTAGCGATCTTCAACAGG	24	(TTA) 7	125-272	ATTO532 (green)
<i>Co166121</i>	F: ACGTCCTCATAAATGCCCAAAC* R: GCAGTGGACTGTAAGGGATG	17	(ATG) 9	220-280	FAM (blue)

2.2.5 Genetic data analysis

The sequences of the ITS rDNA and mtDNA *cox2* obtained for all positive individuals were aligned using the software Clustal X v.2.0 (Larkin et al., 2007), with reference sequences from *C. osculatum* (s.l.) previously deposited in GenBank (Mattiucci et al., 2008). Phylogenetic analysis was carried out by Bayesian inference in MrBayes (Ronquist et al., 2012) based on the concatenated ITS rDNA and mtDNA *cox2* sequences data. The best-fit substitution model was selected using the Bayesian Information Criterion (BIC) and the Akaike Information Criterion (AIC) as implemented in Jmodeltest (Darriba et al., 2012). A run of 1,000,000 iterations was completed, sampling the Markov Chains at interval of 100 generations. The first 20% of the trees were discarded as burn-in, and the remaining were visualized by FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). Posterior probabilities were estimated and used to assess support for each branch in inferred phylogeny with probabilities where $P = 95\%$ being indicative of significant support (Reeder, 2003). The phylogenetic tree was rooted by using *Phocanema decipiens* (s.s.) (MT347695) and *Ascaris suum* (X54253) as outgroups.

The parsimony network for the mtDNA haplotypes was inferred with the software PopART v.1.7 (Leigh and Bryant, 2015) based on the TCS algorithm (Clement et al., 2000).

Analysis of molecular variance (AMOVA) on the mtDNA *cox2* sequences and on SSRs-DNA loci was performed using 1000 permutations with the software ARLEQUIN v.3.5. To investigate the occurrence of population structuring within the genetic datasets of mtDNA *cox2* sequences and nDNA-SSRs loci, a Principal Component Analysis (PCA) was performed in R v.4.3.2 (R Core Team) using the packages adegenet (Jombart, 2008) and ade4 (Dray and Dufour, 2007). The data, consisting of both sequences of mtDNA *cox2* and standardized allele frequencies found at the SSRs loci, were first processed to obtain a genotype matrix. The number of principal components (PCs) was determined by examining the eigenvalues and the explained variance for each PC. The PCA results were visualized in a scatter plot, with the first two PCs (PC1 and PC2) plotted in the axes to highlight the main sources of variation within the dataset, using the package ggplot2 implemented in R v.4.3.2 (Wickham, 2016).

Further, a genetic distance matrix based on the mtDNA *cox2* sequence data was calculated using the ape package (Paradis et al., 2004) implemented in R v.4.3.2 (R Core Team). The genetic distances between all pairs of sequences were used to generate a heatmap, which was clustered hierarchically based on these genetic distances. Genetic differentiation among metapopulations was investigated based on pairwise fixation indices calculated with 1,000 permutations using ARLEQUIN v.3.5 (Excoffier and Lischer, 2010), on genetic data sets acquired from both mtDNA *cox2* sequences and the SSRs loci.

The software Genepop (Rousset, 2008) was used to verify the expected Hardy-Weinberg Equilibrium (HWE) at each SSR locus by exact test outcomes, adjusting the significance level (p) by the Bonferroni correction for multiple tests (Rice, 1989). The inbreeding coefficient F_{IS} was computed for each SSR locus to measure the deficiency (positive values) or excess (negative values) of heterozygotes at each locus, using the software ARLEQUIN v.3.5 (Excoffier and Lischer, 2010).

Genetic diversity inferred from the mtDNA *cox2* data set was estimated at the parameters of: number of haplotypes (N_h), number of unique haplotypes (U), haplotype diversity (H_d), nucleotide diversity (π), average number of nucleotide differences (k), and number of polymorphic sites (S), by DnaSP v.5 (Librado and Rozas, 2009). Genetic variability at the nDNA-SSRs loci was calculated in the same metapopulations of *C. osculatum* sp. B at the following parameters: percentage of polymorphic loci ($P_{99\%}$), alleles richness per locus (A), observed mean heterozygosity (H_O), and expected mean heterozygosity (H_E) by using ARLEQUIN v.3.5.

2.3. Results

2.3.1 Identification of *C. osculatum* (s.l.) specimens by ITS rDNA and mtDNA *cox2*

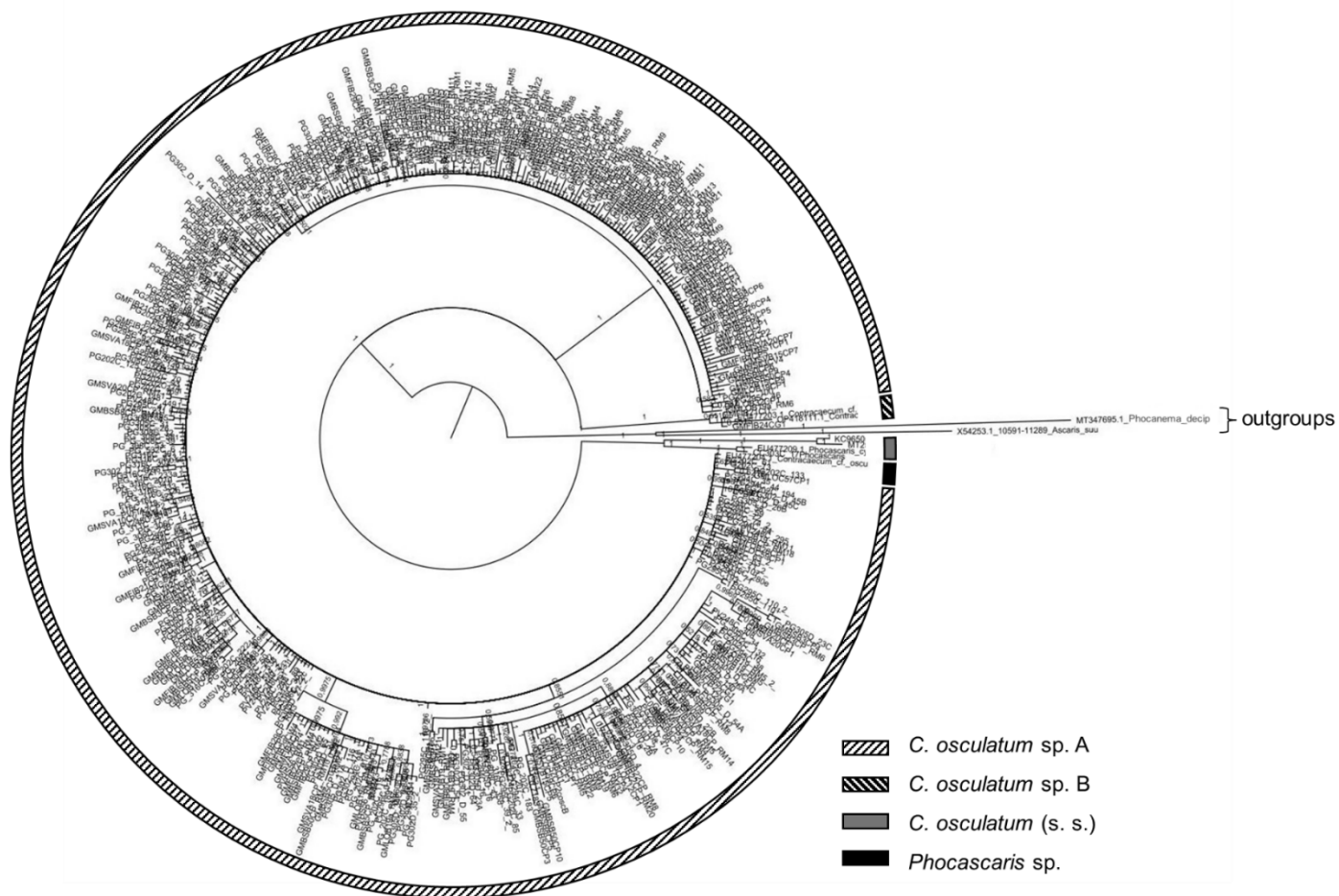
Out of the $N = 388$ historical *C. osculatum* (s.l.) specimens scored, high-quality sequences of the ITS region of rDNA and mtDNA *cox2* genes were obtained for $N = 208$ specimens, allowing species identification (Table 3). Meanwhile, all $N = 214$ individuals from the contemporary *C. osculatum* (s.l.) metapopulations considered in this study, were successfully sequenced at both gene loci (Table 3). Thus, a 436 bp portion of the mtDNA *cox2* gene was successfully obtained from a total of $N = 422$ specimens, including both historical and contemporary nematode specimens. Sequence analysis revealed that $N = 418$ of the sequenced nematodes matched at $>99\%$ with the mtDNA *cox2* sequences deposited in GenBank for *C. osculatum* sp. B. (accession numbers MT448512-19; EU477204) (Table 3); meanwhile, 4 L3 larvae collected from *Gadus morhua* matched at $>99\%$ or 100% with the mtDNA *cox2* sequences deposited in GenBank for *C. osculatum* sp. A. (accession number EU477203) (Table 3).

Similarly, all the $N = 418$ specimens matched at $>99\%$ or 100% with the ITS region of rDNA sequences deposited in GenBank for *C. osculatum* sp. B. Additionally, the 4 specimens assigned to *C. osculatum* sp. A based on mtDNA *cox2*, matched the ITS rDNA sequences of this species available in GenBank (accession numbers AJ250411/AJ250420 and AJ450410/AJ250419, respectively).

The phylogenetic analysis using Bayesian Inference (BI) (Suppl. Fig. 1) on both mtDNA *cox2* and ITS rDNA sequences confirmed this finding, with all N = 208 historical and N = 214 current specimens clustering in a large clade with 100% posterior probability, alongside reference sequences of *C. osculatum* sp. B from GenBank (accession number EU477204) (Suppl. Fig. 1). This clade was distinctly separate from the similarly supported clade (100% posterior probability) in the BI analysis, formed by the 4 *C. osculatum* sp. A specimens found in the current samples, along with the reference specimen of *C. osculatum* sp. A previously deposited in GenBank (accession number EU477203) (Suppl. Fig. 1). The sequences obtained at the ITS rDNA and mtDNA *cox2* were deposited in GenBank under the accession numbers from XXXXXXX to XXXXXXX and from XXXXXXX to XXXXXXX, respectively.

Table 3. Number of specimens assigned to the species *C. osculatum* sp. B or *C. osculatum* sp. A, as inferred from the phylogenetic analysis of the mtDNA *cox2* and the ITS rDNA, reported by sampling period, code area and host species.

Code area	Host species	mtDNA <i>cox2</i> and ITS rDNA			
		1985-86		2021-22	
		<i>C. osculatum</i> sp. B	<i>C. osculatum</i> sp. A	<i>C. osculatum</i> sp. B	<i>C. osculatum</i> sp. A
BE	<i>Pagophilus groenlandicus</i>	24	-	-	-
	<i>Phoca vitulina</i>	12	-	-	-
BS	<i>Pagophilus groenlandicus</i>	92	-	-	-
	<i>Gadus morhua</i>	-	-	60	-
FI	<i>Gadus morhua</i>	-	-	48	2
	<i>Pollachius virens</i>	-	-	10	-
JM	<i>Pagophilus groenlandicus</i>	80			
LO	<i>Gadus morhua</i>	-	-	62	2
SV	<i>Gadus morhua</i>	-	-	30	-
	Tot.	208	-	210	4



Supplementary Figure 1. Circular Bayesian tree inferred from concatenated sequences of ITS rDNA and mtDNA *cox2* obtained from specimens of *C. osculatum* sp. A and *C. osculatum* sp. B analysed in the present study. Evolutionary distance was estimated using the GTR + G and the TN93 + G + I substitution models as implemented in jModeltest (Posada, 2008). Posterior probability values reported at the nodes are the result of 1,000,000 of runs.

2.3.2 Intraspecific genetic variation in mitochondrial mtDNA *cox2* gene

A star-like statistical parsimony network (TCS) (Fig. 2) carried out on the total number (N = 418) of *C. osculatum* sp. B specimens sequenced, was obtained. It illustrates the relationships among the haplotypes found in the two temporal periods (1985-86 and 2021-22). The network is predominantly characterized by numerous unique haplotypes, occurring as singletons, in both the temporal datasets. Specifically, out of a total of N = 312 haplotypes, 130 (approximately 42%) were singletons in the samples collected in 1985-86, and 138 (approximately 44%) were singletons in the samples from the 2021-22 period. Among the haplotypes found in adult specimens (1985-86), the most frequent ones occurred at a frequency of approximately 1.1%, corresponding to an average of two specimens per haplotype. Similarly, in the 2021-22 dataset, the nine most frequent haplotypes occurred at a frequency of approximately 1.2%, averaging three specimens per haplotype. Among the 25 haplotypes, shared across by the two sampling periods, 4 haplotypes (Hap_1, Hap_2, Hap_8, and Hap_39) were the most frequent ones, showing an average frequency of 3%, each represented by an average of 11 specimens; while, the remaining 21 shared haplotypes occurred at an average frequency of 1%, each represented by, approximately, 4 specimens (Fig. 2).

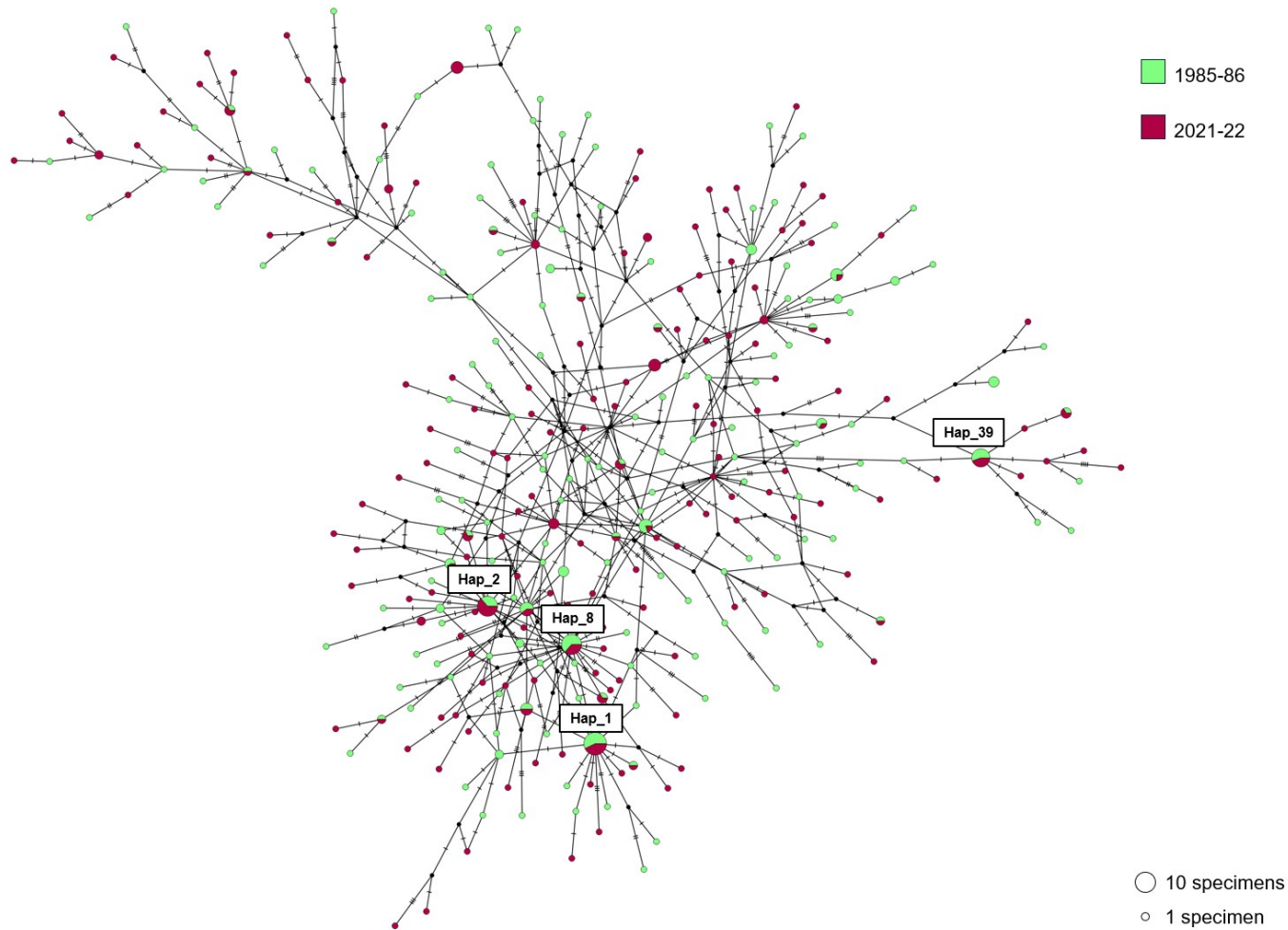


Figure 2. Statistical parsimony network inferred from the TCS algorithm (Clement et al., 2000) using the sequences of the mitochondrial gene *cox2* of specimens of *Contracaecum osculatum* sp. B sampled between 1985-86 and 2021-22. Haplotypes observed in 1985-86 and in 2021-22 are coloured in light green and red, respectively. Each coloured circle represents a unique haplotype, and the size of circles are proportional to the number of individuals. The most frequent haplotypes shared over temporal scaled, are labelled.

The AMOVA analysis based on mtDNA *cox2* showed that both in historical and current metapopulations of *C. osculatum* sp. B, most of the variance was significantly allocated within the analysed metapopulations (with percentage over 99%), rather than among them (Table 4a).

Table 4a. Analysis of molecular variance (AMOVA) between and within metapopulations of the species *C. osculatum* sp. B, using genetic data sets obtained from mtDNA *cox2*.

		Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
mtDNA <i>cox2</i>	1985-86	Among populations	1	4.082	0.005	0.15
		Within populations	206	727.701	3.532	99.85
		Total	207	731.784	3.537	
	2021-22	Among populations	3	3.770	0.005	0.12
		Within populations	206	766.140	3.683	99.88
		Total	209	769.910	3.687	

The heatmap analysis based on genetic distance data from mtDNA *cox2* sequences, carried out on both historical and contemporary specimens (Fig. 3), revealed the existence of two main clusters, hereafter indicated as Pop 1 and Pop 2 (Fig. 3). A significant genetic differentiation was observed between Pop 1 and Pop 2, with an average $F_{st} = 0.262$ ($p \ll 0.001$). While lower F_{st} values were observed within Pop 1 (F_{st} value close to zero, $p = 0.405 \pm 0.005$), and Pop 2 (F_{st} value close to zero, $p = 0.814 \pm 0.004$).

The Principal Component Analysis (PCA) performed on both historical and contemporary samples, (Fig. 4), showed a consistent clustering pattern with the detection of the two main Pop 1 and Pop 2 clusters. Notably, the contemporary sample, formed by the larval specimens, predominantly clustered within Pop 2 (Fig. 4).

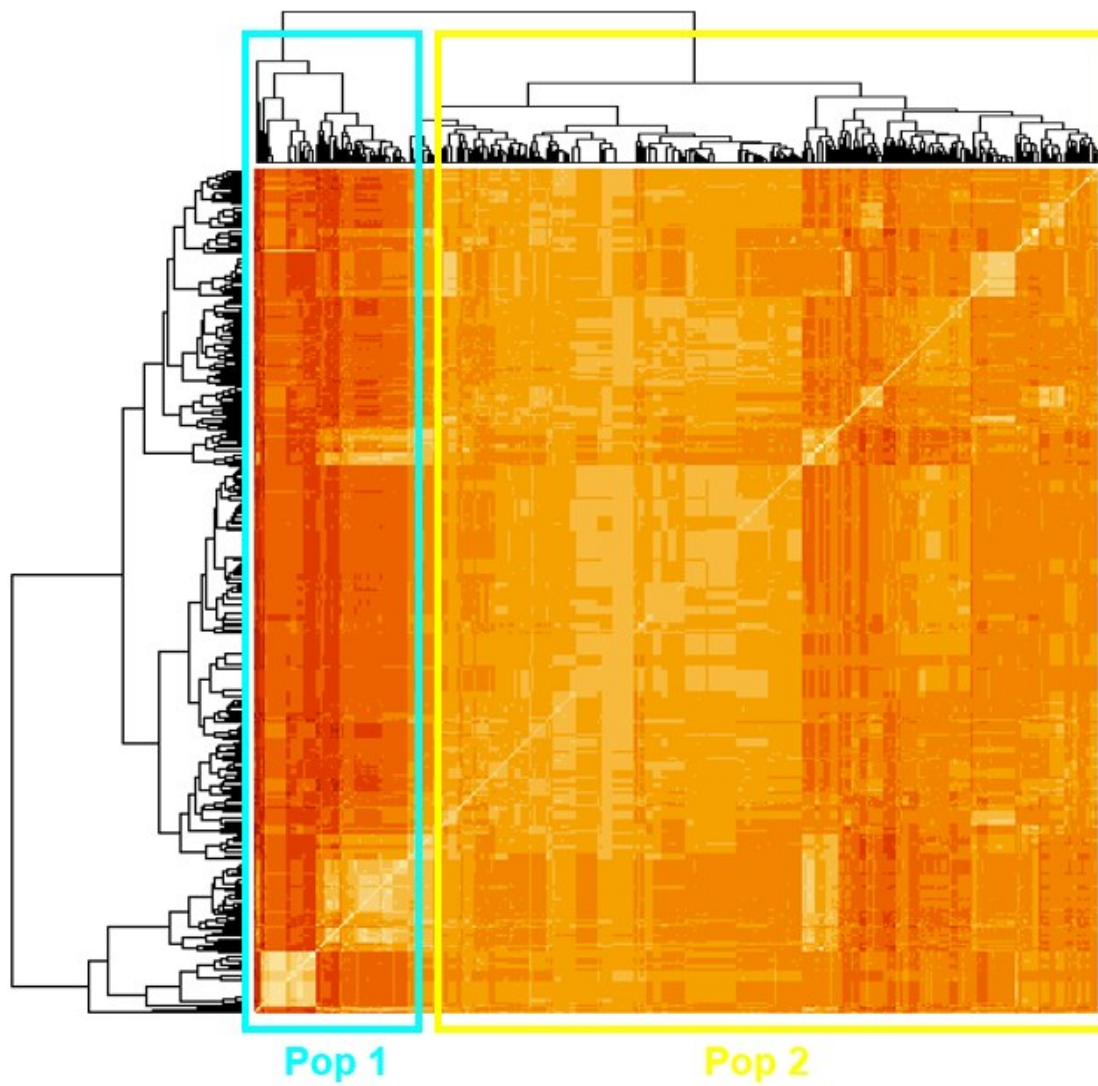


Figure 3. Heatmap displaying pairwise genetic distance values inferred from mtDNA *cox2* sequences from specimens of *C. osculatum* sp. B (both historical and current samples). Rows and columns represent individual samples, clustered hierarchically based on their pairwise genetic distance values. The existence of two primary clusters is evident, likely corresponding to a substructuring of *C. osculatum* sp. B, here named as Pop 1 (light blue) and Pop 2 (yellow), respectively.

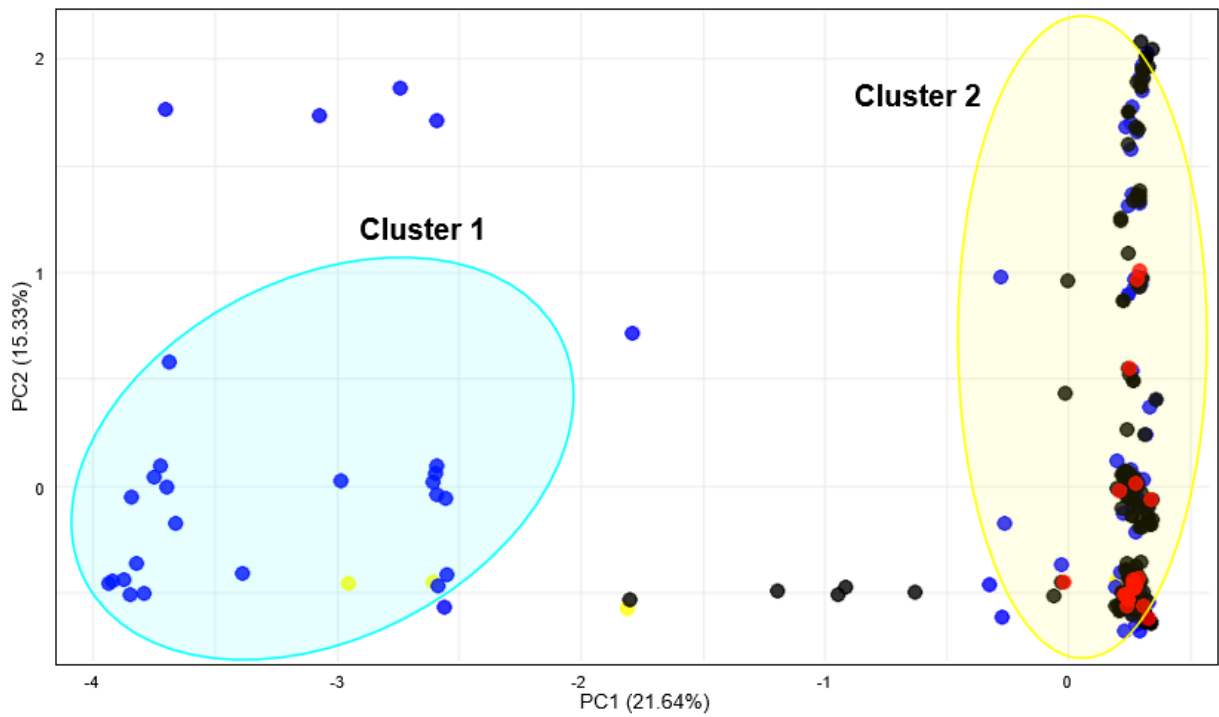


Figure 4. Scatter plot of the Principal Component Analysis (PCA) performed on mtDNA *cox2* sequences from adult and larval specimens of *C. osculatum* sp. B. The first two principal components explained 21.64% (PC1) and 15.33% (PC2) of the variance, with PC1 accounting for the separation between these two groupings. Colored clusters represent the Pop 1 (light blue) and Pop 2 (yellow), respectively. Red and yellow dots correspond to specimens of *C. osculatum* sp. B sampled from the harp seals and the harbour seal in the South Norwegian Sea, respectively, while blue dots represent specimens collected from harp seals the northern Barents-White Sea and the Jan Mayen area (see Table 1 for sampling locations). Black dots represent larval specimens sampled in fish hosts.

2.3.3 Intraspecific genetic variation in the nuclear SSRs loci

The total number of *C. osculatum* sp. B specimens genotyped at the seven scored SSRs DNA loci was N = 418, including historical (N = 208) and current samples (N = 210) (Table 3). The AMOVA analysis, based on six nuclear SSRs-DNA loci, revealed that in both historical and current metapopulations of *C. osculatum* sp. B, most of the genetic variance was significantly allocated within the metapopulations ($\approx 91\%$ and $\approx 77\%$, respectively), rather than among them (Table 4B). Notably, a significant variation was found among individuals within the current metapopulations ($\approx 22\%$) (Table 4B), with $F_{IT} = 0.17$ ($p < 0.05$) (data not shown).

Table 4b. Analysis of molecular variance (AMOVA) between and within metapopulations of the species *C. osculatum* sp. B, using genetic data sets obtained from six SSRs loci

		Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
6 SSRs loci	1985-86	Among populations	1	1.728	0.028	1.20
		Among individuals within population	206	450.287	0.180	7.77
		Within individuals	208	391.000	2.210	91.03
		Total	415	843.015	2.418	
	2021-24	Among populations	3	18.210	0.030	1.05
		Among individuals within population	206	634.996	0.608	21.76
		Within individuals	210	417.000	2.152	77.18
		Total	419	1070.206	2.790	

The seven microsatellite loci scored were polymorphic in *C. osculatum* sp. B, with the total number of alleles ranging from 5 (observed at the locus *Co121208*) to 18 (found at the locus *Co210259*) (Table 5A). Deviations from Hardy-Weinberg Equilibrium (HWE) at each locus were assessed in the selected populations of both species (Table 5A). Positive values of F_{IS} indicated an excess of homozygous genotypes at the selected loci, while negative values suggested an excess of heterozygotes compared to the expected HWE (Fig. 5A). No significant deviations were found between observed (HO) and expected (HE) heterozygosity at six SSRs loci, in both historical and current *C. osculatum* sp. B populations. However, the locus *Co121208* showed significant

deviations from HWE in both historical and contemporary metapopulations (Table 5A). This locus exhibited a marked excess of homozygotes in all metapopulations across the temporal scale (Table 5A), with a positive FIS value (Fig. 5A). Interestingly, when splitting the genotypes of the historical adult specimens of *C. osculatum* sp. B by sex, male worms (N=18) were homozygous at this locus (FIS = 1) (Fig. 5B). In contrast, no significant departures from HWE were found (Table 5B) with a FIS value near to 0 (Fig. 5B), in the female specimens. These results suggest that the *Co121208* locus is sex-linked locus. Thus, it was excluded from the allele frequencies estimates formed by the contemporary larval population (Suppl. Table 1). While, in the case of historical adult sample, alleles frequencies were calculated only on adult females (N=34) (Suppl. Table 1).

Table 5a. Hardy Weinberg equilibrium observed at 7 SSRs DNA loci in the metapopulations of *C. osculatum* sp. B from the two different periods (1985-86 vs 2021-22). N, number of individuals of *C. osculatum* sp. B studied at each DNA SSRs locus; H_O , observed heterozygosity; H_E , expected heterozygosity; p , significance ($p < 0.05$) of the deviation from HWE expectation; A, number of alleles and (U), number of unique alleles occurring at frequencies > 0.1 .

		1985-86	2021-22
Locus			
<i>Co42335</i>	N	208	210
	H_O	0.69	0.69
	H_E	0.67	0.70
	p value	0.46	0.19
	A (U)	10(3)	7(0)
<i>Co215165</i>	N	208	210
	H_O	0.65	0.62
	H_E	0.68	0.66
	p value	0.55	0.08
	A (U)	10(3)	8(1)
<i>Co97601</i>	N	208	210
	H_O	0.73	0.74
	H_E	0.77	0.78
	p value	0.11	0.10
	A (U)	10(3)	7(0)
<i>Co85484</i>	N	208	210
	H_O	0.83	0.76
	H_E	0.90	0.90
	p value	0.15	0.06
	A (U)	17(1)	18(2)
<i>Co121208</i>	N	208	210
	H_O	0.42	0.43
	H_E	0.68	0.69
	p value	***	***
	A (U)	5(0)	5(0)
<i>Co210159</i>	N	208	206
	H_O	0.83	0.80
	H_E	0.91	0.91
	p value	0.06	0.06
	A (U)	18(2)	16(0)
<i>Co166121</i>	N	208	206
	H_O	0.75	0.75
	H_E	0.83	0.82
	p value	0.06	0.06
	A (U)	14(4)	10(0)

Table 5b. Hardy Weinberg equilibrium observed at the sex-linked locus, i.e. *Co121208*, in adult male and female specimens of *C. osculatum* sp. B sampled in 1985-86. N, number of individuals of *C. osculatum* sp. B studied at the present SSRs locus; H_O , observed heterozygosity; H_E , expected heterozygosity; p , significance ($p < 0.05$) of the deviation from HWE expectation.

		♀♀	♂♂
Locus			
<i>Co121208</i>	N	34	18
	H_O	0.56	0.00
	H_E	0.66	0.65
	p value	0.43	***

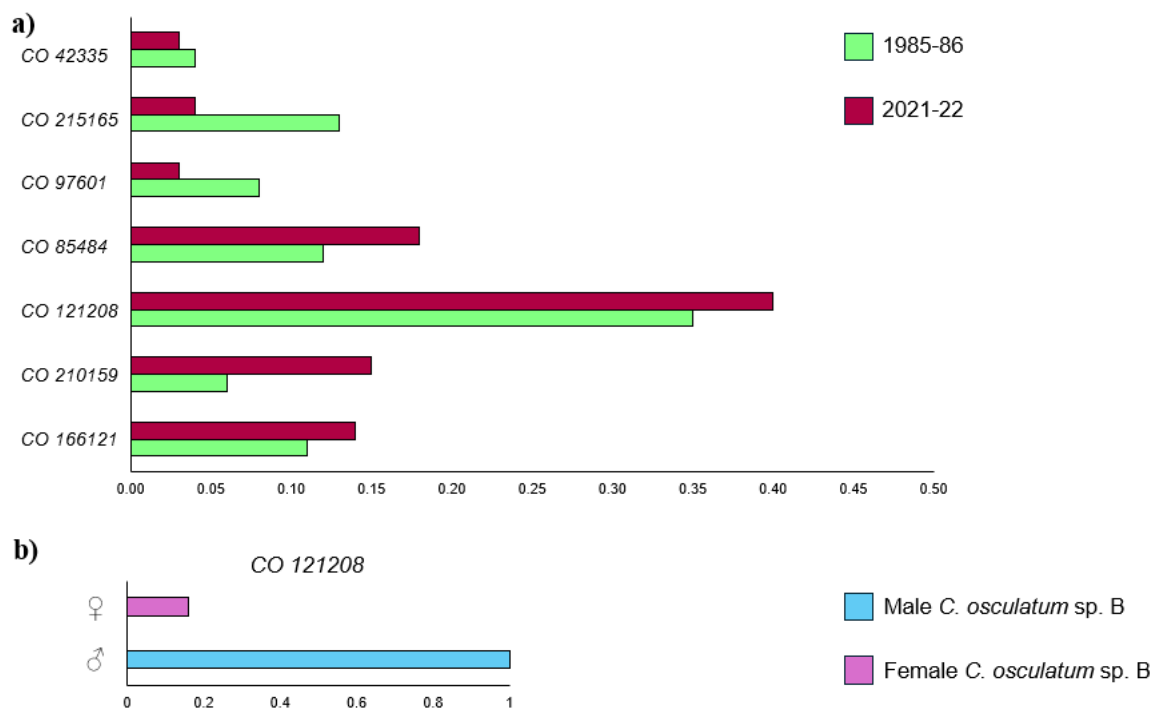


Figure 5. a) FIS calculated at the seven microsatellites loci studied in the species. Negative values indicate heterozygous excess while positive values indicate homozygous excess from that expected under Hardy–Weinberg Equilibrium (HWE), and b) FIS in male and female adult specimens of *C. osculatum* sp. B at the sex-linked locus *Co121208*.

Supplementary Table 1. Allele frequencies, occurring at frequency ≥ 0.01 , observed at six microsatellite loci tested in populations of *C. osculatum* sp. B sampled in the two different temporal periods (1985-86 and 2021-22). As regards the sex-linked locus (i.e. *Co121208*) the most reliable estimate of allele frequencies was calculated in adult population according to the sex-linked genetic model estimate, assuming: (i) the hemizyosity of the males at that locus; (ii) their adult female counterparts, as biallelic at the sex-linked locus.

Locus	Alleles	1985-86	2021-22
<i>Co42335</i>	65	0.01	-
	68	0.01	0.01
	71	0.04	0.03
	74	0.17	0.22
	77	0.50	0.41
	80	0.20	0.25
	83	0.04	0.06
	86	0.01	0.02
	89	0.01	-
	95	0.01	-
<i>Co215165</i>	110	0.01	0.03
	114	0.46	0.46
	118	0.28	0.28
	122	0.13	0.09
	126	0.06	0.03
	130	0.02	0.06
	134	0.01	0.02
	138	-	0.02
	142	0.01	-
	146	0.01	-
	154	0.01	-
<i>Co97601</i>	107	0.01	-
	137	0.01	-
	140	0.06	0.08
	143	0.24	0.22
	146	0.30	0.33
	149	0.24	0.21
	152	0.08	0.10
	155	0.03	0.03
	158	0.02	0.02
	164	0.01	-
	167	-	-
<i>Co85484</i>	131	-	0.01
	137	0.01	0.03
	140	0.03	0.05

	<i>143</i>	0.07	0.08
	<i>146</i>	0.06	0.08
	<i>149</i>	0.10	0.03
	<i>152</i>	0.16	0.13
	<i>155</i>	0.13	0.11
	<i>158</i>	0.13	0.07
	<i>161</i>	0.09	0.13
	<i>164</i>	0.09	0.06
	<i>167</i>	0.04	0.06
	<i>170</i>	0.02	0.03
	<i>173</i>	0.03	0.07
	<i>176</i>	0.01	0.02
	<i>179</i>	0.01	0.02
	<i>182</i>	0.01	0.01
	<i>185</i>	-	0.01
	<i>191</i>	0.01	-
Co121208	<i>160</i>	0.18	na
	<i>163</i>	0.44	na
	<i>166</i>	0.33	na
	<i>169</i>	0.05	na
Co210159	<i>203</i>	0.01	0.03
	<i>206</i>	0.02	0.05
	<i>209</i>	0.07	0.05
	<i>212</i>	0.17	0.15
	<i>215</i>	0.13	0.11
	<i>218</i>	0.09	0.10
	<i>221</i>	0.07	0.09
	<i>224</i>	0.10	0.08
	<i>227</i>	0.07	0.11
	<i>230</i>	0.06	0.05
	<i>233</i>	0.06	0.05
	<i>236</i>	0.04	0.05
	<i>239</i>	0.04	0.03
	<i>242</i>	0.03	0.02
	<i>245</i>	0.01	0.01
	<i>248</i>	0.01	0.01
	<i>251</i>	0.01	-
	<i>257</i>	0.01	-
Co166121	<i>220</i>	0.01	0.01
	<i>223</i>	0.01	-
	<i>226</i>	0.01	-
	<i>229</i>	0.12	0.13
	<i>232</i>	0.11	0.04

<i>235</i>	0.04	0.05
<i>238</i>	0.04	0.08
<i>241</i>	0.07	0.16
<i>244</i>	0.32	0.34
<i>247</i>	0.15	0.09
<i>250</i>	0.07	0.07
<i>253</i>	0.03	0.02
<i>256</i>	0.01	-
<i>259</i>	0.01	-

Principal Component Analysis (PCA) based on allele frequencies at the six SSRs DNA loci for the full dataset (N = 418 specimens), revealed a separation along the X-axis, forming two distinct clusters indicated as well as Pop 1 and Pop 2 (Fig. 6). Slightly significant F_{st} value based on the SSRs-DNA loci was found between Pop 1 and Pop 2 ($F_{st} = 0.04$, $p = 0.05 \pm 0.235$). Whereas, no significant genetic differentiation was observed when comparing the specimens within the same Pop (data not shown). Similarly to the PCA based on mtDNA *cox2*, most of the contemporary specimens formed by L3 stage larvae, grouped within the Pop 2 (Fig. 6).

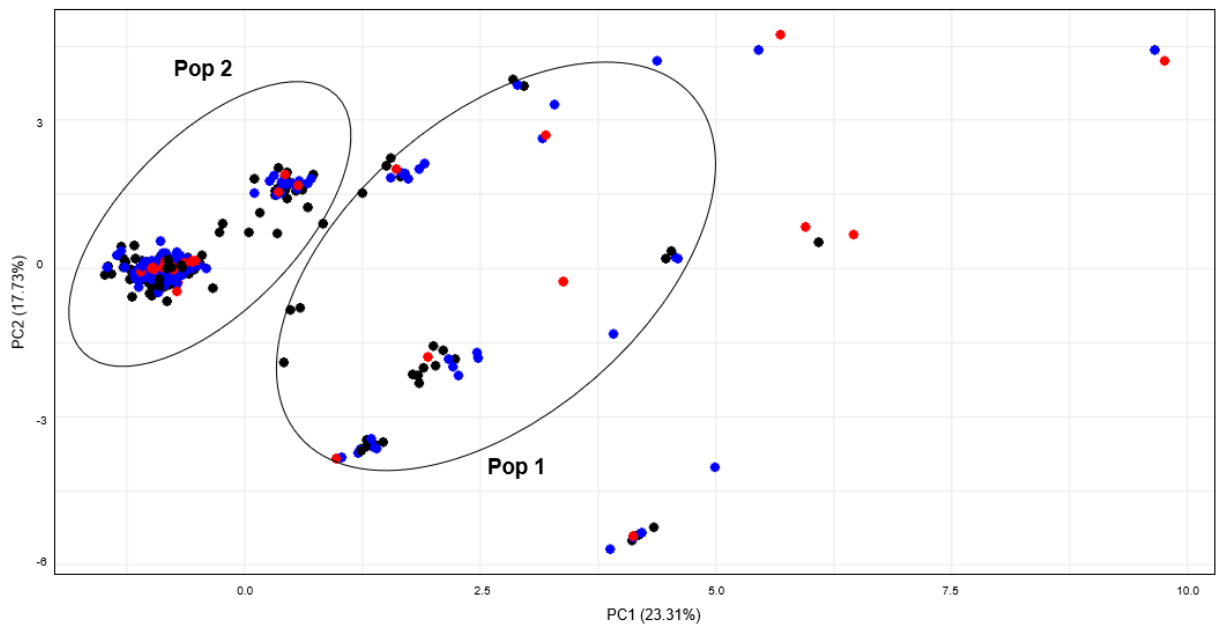


Figure 6. Scatter plot of the Principal Component Analysis (PCA) performed on the microsatellite (SSRs) dataset, including both adult and larval specimens of *C. osculatum* sp. PC1 explains 23.31% of the variance, while the Y-axis accounted for a 17.73%. Colors indicate specific host associations: red dots correspond to sampled from seals in the South Norwegian Sea, while blue dots represent specimens collected from the northern Barents-White Sea and the Jan Mayen area (see Table 1 for sampling locations). Black dots represent individuals sampled from fish hosts.

2.3.4 Intraspecific genetic variability at mitochondrial (mtDNA *cox2*) level

The estimates of genetic variability parameters, based on the temporal datasets of mtDNA *cox2* sequences obtained from *C. osculatum* sp. B specimens, are summarized in Table 6A. A total of N= 165 haplotypes were identified among specimens sampled between 1985-86. Out of these, 142 (approximately 86%) occurred once. Whereas N = 172 haplotypes were identified from current samples dating from 2021-22, with 152 singletons (approximately 88%).

Within the Pop 1, a total of 30 haplotypes of mtDNA *cox2* were identified in 36 specimens of *C. osculatum* sp. B sampled during 1985–1986, whereas 136 haplotypes were detected in 172 individuals from the Pop 2 for the same period. Among the latter, 28 (approximately 93%) and 118 (approximately 87%) were singletons, respectively. In the contemporary parasite sample (2021-2022), 32 mtDNA *cox2* haplotypes were identified in 40 specimens of *C. osculatum* sp. B from pop 1, with 28 unique haplotypes (approximately 86%). Meanwhile, 140 haplotypes were detected in 170 individuals from Pop 2, of which 125 were singletons (approximately 89%) (Table 6A).

The overall values of nucleotide diversity (π) and haplotype diversity (Hd) observed in the two temporal periods are shown in Table 6. Lower nucleotide diversity (π) values were observed in the Pop 1 sampled in 2021-22 ($\pi = 0.021 \pm 0.001$) compared to the 1985-86 samples ($\pi = 0.023 \pm 0.001$). Meanwhile, Pop 2 exhibited similar values ($\pi = 0.013 \pm 0.001$) between the two sampling periods. Overall, the observed nucleotide diversity ($\pi = 0.017 \pm 0.001$) was similar across both temporal samples of the parasite species (Table 6A).

2.3.5 Intraspecific genetic variability at nuclear (SSRs-DNA loci) level

Estimates of genetic variability at six SSRs-DNA loci (excluding the sex-linked locus Co121208), based on the temporal and spatial dataset defined by the two clusters, Pop 1 and Pop 2, are presented in Table 6B. The six microsatellite loci were polymorphic across all the samples analyzed. The average number of alleles (A) ranged from A = 8.0 in Pop 1 (1985-86) to A = 13.5 in Pop 2 (1985-86). The lowest value of observed heterozygosity (HO) was recorded in Pop 2 sampled in 2021-22 (HO = 0.66). Overall, genetic variability was higher in the samples collected between 1985-86 (HO = 0.73) compared to the current larval sample of *C. osculatum* sp. B from 2021-22 (HO = 0.63). Similarly, the contemporary sample showed a slightly lower mean number of alleles (A = 11.0) compared to the 1985-86 samples (A = 13.7) (Table 6B).

Table 6. a) Genetic variability estimates as inferred from mtDNA *cox2* sequences analysis of metapopulations of *C. osculatum* sp. B sampled in 1985-86 and in 2021-22, defined by Principal Component Analysis (PCA), and overall. N, number of considered sequences; Nh, number of haplotypes; U, number of unique haplotypes; π , nucleotide diversity; Hd, haplotype diversity; k, average number of nucleotide differences; S, number of polymorphic sites. **b)** Genetic variability values at the parameters, as inferred from 6 polymorphic DNA SSRs loci analysis of metapopulations of *C. osculatum* sp. B sampled in 1985-86 and in 2021-22, defined by Principal Component Analysis (PCA), and overall. N, number of analysed specimens; n, number of SSRs loci considered; P₉₉, proportion of polymorphic loci at 99.0% criterion; A, average number of alleles per locus; H_O, mean observed heterozygosity per locus.

		Pop 1		Pop 2		Overall	Overall
		1985-86	2021-22	1985-86	2021-22	1985-86	2021-22
A) mtDNA <i>cox2</i>							
	N	36	40	172	170	208	210
	Nh (U)	30 (28)	32 (28)	136 (118)	140 (125)	165 (142)	172 (152)
	$\pi \pm SD$	0.023 $\pm$ 0.001	0.021 $\pm$ 0.001	0.013 $\pm$ 0.001	0.013 $\pm$ 0.000	0.017 $\pm$ 0.000	0.017 $\pm$ 0.001
	Hd $\pm$ SD	0.979 $\pm$ 0.016	0.982 $\pm$ 0.012	0.994 $\pm$ 0.002	0.996 $\pm$ 0.002	0.996 $\pm$ 0.001	0.997 $\pm$ 0.001
	k	10.347	9.570	5.662	5.848	7.070	7.367
	S	64	60	86	99	107	113
B) 6 SSRs loci							
	N	36	40	172	170	208	210
	n	6	6	6	6	6	6
	P ₉₉	100	100	100	100	100.0	100.0
	A	8.0	9.3	13.5	11.5	13.5	11.5
	H _O	0.72	0.73	0.74	0.66	0.73	0.63
	H _E	0.79	0.81	0.80	0.80	0.80	0.80

Histograms in Fig. 7 illustrate the overall temporal variation in standardized allelic frequencies of *C. osculatum* sp. B by comparing those from historical and current metapopulations. Several rare alleles present in the SSRs DNA loci in the historical population were missing in the current population (Fig. 7). For example, alleles 251 and 257 at the locus *Co210159*, and alleles 223, 226, 256, and 259 at the locus *Co166121* are missing. Additionally, other SSRs loci showed a lower frequency in the current population compared to the historical sample (Fig. 7). Conversely, new rare alleles appear to be present in the current populations, such as those found at the loci *Co215165* (allele 138) and *Co85484* (alleles 131 and 185), which were not observed in the historical sample (Fig. 7).

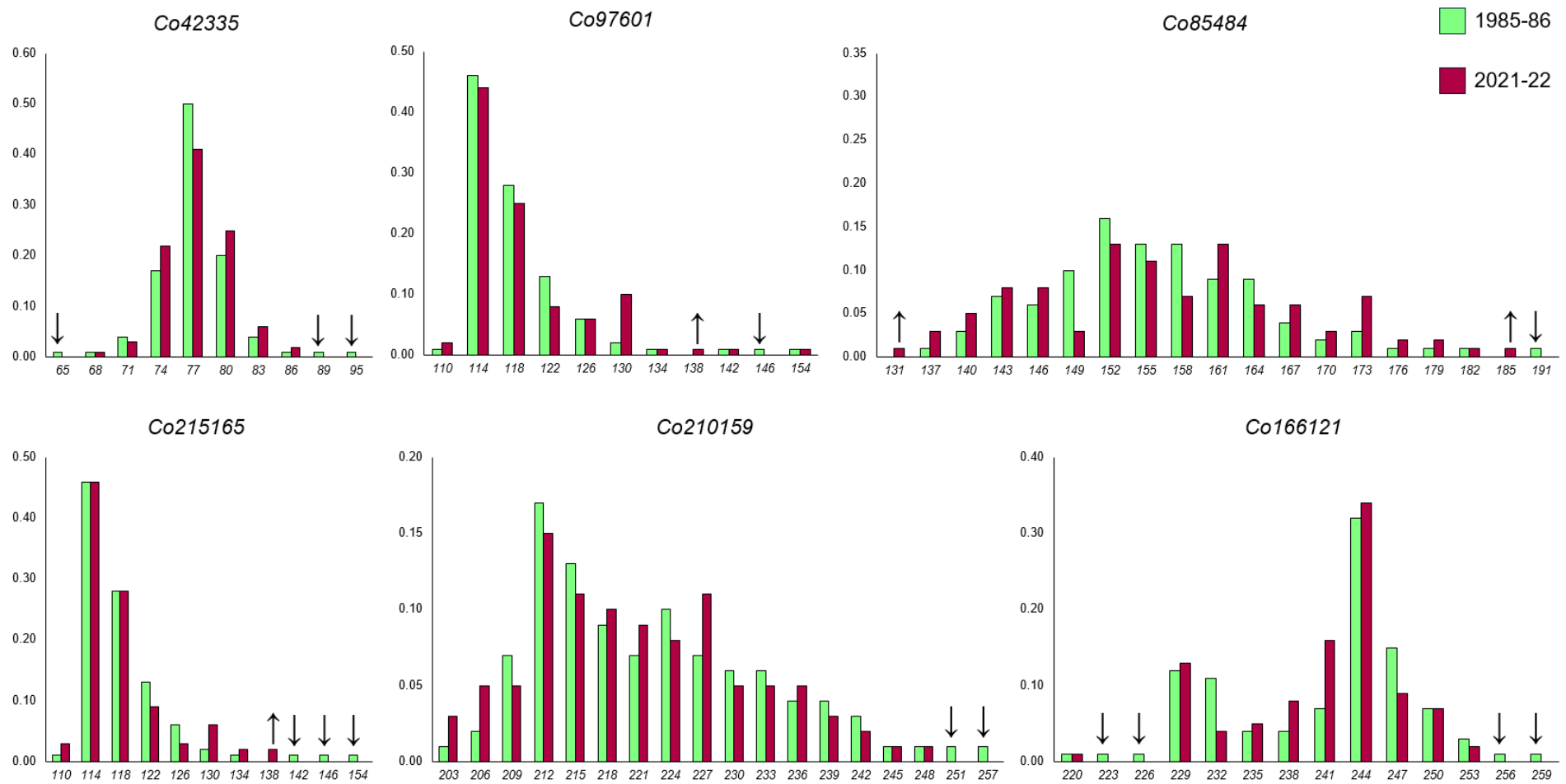


Figure 8. Histograms illustrating the allele frequencies observed at six microsatellite loci (excluding the sex-linked locus *Co121208*) in *C. osculatum* sp. B over temporal scale (1985-86 and 2021-22). Each bar represents the frequency (Y-axis) of an allele at a given locus (X-axis). ↓ indicates apparent lost alleles, while ↑ indicates new observed alleles.

2.4 Discussion

This study provides the first analysis of population genetic structure and an estimation of genetic variability over a three-decade temporal scale for the Arctic anisakid nematode *C. osculatum* sp. B. Most population genetics studies infer microevolutionary mechanisms based on data from a single time point. However, to directly observe dynamic processes, it is crucial to conduct temporal population surveys. Few molecular studies on parasites and vectors have analyzed population genetic structure across multiple temporal points. These studies often employ various markers, including SSRs-DNA loci and mitochondrial DNA, and primarily focus on nematode model species such as *Caenorhabditis elegans* (Barrière and Félix, 2007) or on the malaria parasite *Plasmodium falciparum* (Niang et al., 2017; Huang et al., 2018) and its mosquito vectors (Lukindu et al., 2018). Generally, studies on the population genetic structure over a temporal scale are lacking for marine parasites (rev. in Mattiucci et al., 2025). Previous population genetic analyses of anisakid nematodes, including comparisons with abundance estimates over time, were performed in the Antarctic members of *C. osculatum* (s.l.), specifically *C. osculatum* sp. D and *C. osculatum* sp. E. These analyses revealed that both the high genetic variability and parasitic load (as a proxy for population size) of these Antarctic worms remained stable over two decades (Mattiucci et al., 2015). This finding was attributed to the large population size of both Antarctic seals (*Leptonychotes weddellii*) and fish hosts which serve as definitive and intermediate/paratenic hosts for the parasites, respectively. It has been thus suggested that the stability of the trophic web connecting these hosts contributes to maintain a high parasite transmission rate in the Antarctic region (Mattiucci et al., 2015).

2.4.1 Novel nuclear polymorphic loci in *C. osculatum*

This study highlights the utility of polymorphic loci in population genetics by combining mitochondrial and nuclear markers to capture fine-scale patterns of parasite genetic variation, providing a comprehensive view of population structure, even across a temporal scale. In particular, the development and validation of SSRs DNA markers for *C. osculatum* sp. B represents a significant methodological advancement. The use of microsatellites in anisakid parasites has proven to be an invaluable tool for understanding their population dynamics, genetic structure, and evolutionary processes. Previous studies have successfully applied SSRs DNA loci to investigate various genetic aspects of marine parasites, providing insights into their population structure, genetic diversity, and phenomena such as hybridization and introgression. However, the application of SSRs DNA markers in parasitic nematodes has been relatively limited (Betson et al., 2011; Patrelle et al., 2014; Rabelo et al., 2017; Greeff et al., 2018). Jossart et al. (2017) used

ten microsatellite loci to explore patterns of population structure in the parasitic species *Dissodactylus primitivus* and its host, the heart urchin. The population genetic structure of *Lepeophtheirus salmonis*, an ectoparasite of Atlantic and Pacific salmonids, has been studied through the analysis of microsatellite DNA variation among wild and farmed hosts (Todd et al., 2004). Additionally, Criscione and Blouin (2007) employed microsatellite analysis of trematode parasites to trace the origin of the steelhead trout *Oncorhynchus mykiss*. In the case of anisakid nematodes, SSRs DNA loci were successfully developed and validated in the three species of the *Anisakis simplex* (s. l.) complex (Mladineo et al., 2017; Mattiucci et al., 2019; Bello et al., 2020). These loci have proven to be of diagnostic value in identifying the parental genome of the three species and correctly classifying hybrid categories between *A. pegreffii* and *A. simplex* (s. s.) (Mattiucci et al., 2025), as well as F1 hybrids between *A. pegreffii* and *A. berlandi* (Bello et al., 2021).

In the present study, seven loci were successfully validated through robust amplification from individual worms, providing clear and unambiguous genotype detection across a high number of specimens (N= 418) of *C. osculatum* sp. B. These markers can thus be used in future population genetics studies of this species, sampled in other geographical areas, as well as in other species of the *C. osculatum* (s. l.) complex.

In *C. osculatum* sp. B, the microsatellite markers exhibited a high level of polymorphism, with expected heterozygosity (H_E) values ranging from $H_E = 0.67$ at the locus *Co215165* to $H_E = 0.91$ at the locus *Co210159*, showing an overall average of $H_E = 0.78$. The average number of alleles per locus was $A = 11.1$, slightly higher than the value of 10.8 observed for the microsatellites developed for the *A. simplex* (s.s.) complex (Mattiucci et al., 2019; Bello et al., 2020, 2021). The high polymorphism observed suggests that the microsatellites developed in this study are valuable tools for fine-scale genetic studies, including assessments of population differentiation, gene flow, genetic diversity, and the detection of genetic erosion occurrence in the gene pools of these marine anisakid nematodes.

Additionally, an interesting finding in the SSRs DNA loci was the discovery of a sex-linked locus in *C. osculatum* sp. B. Sex-linked loci have been previously reported in other anisakid nematodes, such as species within the *A. simplex* (s.l.) complex (Mattiucci et al., 2019, 2025; Bello et al., 2020, 2021). Sex-linked SSR DNA loci have also been identified in *A. lumbricoides*, where they may constitute around 20% of its genome. According to Mattiucci et al. (2019, 2025), these loci represent approximately 33% of the SSRs loci in *A. simplex* (s.l.). The discovery of sex-linked SSR markers in anisakid nematodes could be instrumental for accurate sex assignment at any life-history stage of these parasites.

2.4.2 Population genetic structure of *C. osculatum* sp. B

Despite the clear panmixia of the parasite species studied, as highlighted by the star-like structuring of the haplotype network (Fig. 2), both PCA and heatmap analyses of the mtDNA *cox2* and SSR loci datasets indicated the existence of two clusters with a significant observed genetic differentiation value (on average, $F_{st} = 0.26$ based on mtDNA *cox2*). This finding suggests the presence of a certain level of genetic substructuring among the adult populations of *C. osculatum* sp. B in the study area. Such values of genetic differentiation at the mitochondrial level have been previously observed between conspecific populations of other anisakids (Mattiucci and Nascetti, 2008; Mattiucci et al., 2025).

It has been also postulated that population genetic structure of definitive hosts participating in the life-cycle of anisakids may be a driver in shaping the population structure of their endoparasites (Mattiucci et al., 2025). Interestingly, the existence of these two subpopulations of the parasite species appears to be linked to the intraspecific population genetic structuring of its main definitive host in the study area, the harp seal (*Pagophilus groenlandicus*). Previous studies on the population genetic structure of harp seal populations, inferred from mitochondrial *cytb* gene sequences, revealed significant genetic differentiation between Northwest and Northeast Atlantic populations (Perry et al., 2000). Additionally, distinct populations of the harp seal have been identified based on their breeding grounds (i.e., Northwest Atlantic, Greenland Sea, and White Sea–Barents Sea) (Folkow et al., 2004; Nordøy et al., 2008). The population genetic structure of *C. osculatum* sp. B in the historical sample, appears to mirror that of its definitive hosts, primarily the harp seal, from which the adults were collected. This finding may result from host-parasite microevolutionary co-phylogeographical events in the Arctic Sea waters. Co-phylogenetic patterns between pinniped hosts and their anisakid endoparasites in the genus *Contracaecum* have been proposed based on the comparison of phylogenetic analyses of the two interacting groups (pinnipeds and *Contracaecum* spp. maturing in seal hosts) (Mattiucci and Nascetti, 2008). These patterns suggest that the anisakids have co-evolved with their definitive hosts throughout evolutionary history. Thus, the harp seal populations, from which the parasite was collected, may be a source of genetic variation, as indicated by the clustering patterns here observed, while the main fish hosts may contribute to maintaining high gene flow between the populations of *C. osculatum* sp. B.

Indeed, the contemporary populations of *C. osculatum* sp. B analyzed, consisting of larval specimens sampled from fish hosts — primarily *Gadus morhua* (cod) — did not show clear distinctiveness between two clusters. Most of them likely clustered within the Pop 2 (Fig. 4). It would be reasonable to hypothesize that the fish hosts of the parasite species likely play an important role in maintaining the gene flow between the metapopulations of *C. osculatum* sp. B in

Arctic waters. This could be the consequence of their high mobility and dispersion in the Arctic/Sub-arctic ecosystems. Indeed, the role of fish hosts in facilitating genetic homogenization should not be overlooked. The panmixia observed in this study in *C. osculatum* sp. B (Fig. 2) likely reflects ecological connectivity driven by trophic transmission, with fish serving as a driver of genetic exchange among geographically dispersed definitive host populations in Arctic and sub-Arctic waters. The gene flow between the parasite metapopulations may be due to the long-distance migratory behaviour of the North East Atlantic Cod "ecotype" (indicated as NEAC) (Dahle et al., 2018), which occasionally migrates northward (Bogstad, 2023).

Overall, these findings suggest that the population genetic structure of *C. osculatum* sp. B might be shaped by both seal definitive and fish intermediate/paratenic host population structures. This supports the idea that this parasite species may also reflect the structure of the trophic web in Arctic sea waters, including seals and fish as key hosts in its life cycle.

2.4.3 Temporal genetic variability in *C. osculatum* sp. B

The analysis of mtDNA *cox2* revealed substantial changes in genetic variability over the temporal scale. In the historical sample, a high number of unique haplotypes were identified, many of which were not found in the contemporary samples. Specifically, the two temporal samples exhibited a high proportion of unique haplotypes, 86% and 88%, respectively. This pattern is further supported by the TCS haplotype network (Fig. 2) and illustrated by histograms representing haplotype frequency variations over time, which also highlight a limited number of shared variants between the two temporal samples considered here (Fig. 8). The apparent absence of certain rare haplotypes in the contemporary sample, compared to the historical one, may indicate a loss of genetic variability due to genetic drift, driven by fluctuations in host populations or environmental changes that directly affect the free-living larval stages. Conversely, the appearance of new haplotypes at the mtDNA *cox2* locus, such as those observed in the current samples, suggests ongoing microevolutionary processes. These findings suggest a dynamic process of haplotype turnover, where rare haplotypes may have been lost over time, only to be replaced by new variants (Fig. 8).

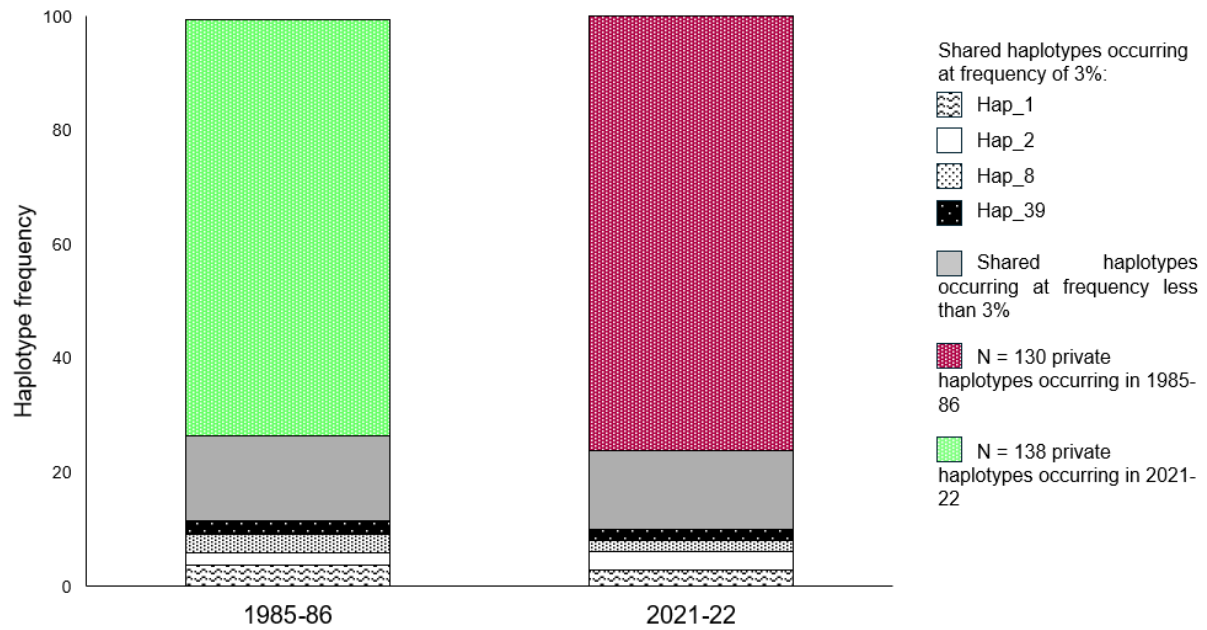


Figure 8. Temporal variation in haplotype frequencies observed in the two datasets (1985-86 and 2021-22). The most frequent haplotypes shared over temporal scale occurring at frequency $>3\%$, i.e., Hap_1, Hap_2, Hap_8, Hap_39, are represented (see Legend for the specific colour patterns). Haplotypes shared over temporal scale occurring at frequency $<3\%$ are coloured in grey. Private haplotypes occurring at frequency $<3\%$ in 1985-86 and in 2021-22 are coloured in light green and red, respectively.

This turnover could help explain the overall similarity in nucleotide and haplotype diversity between the two temporal datasets. It can be speculated that this process reflects demographic shifts in the parasite population, potentially driven by fluctuations in both its intermediate and definitive hosts (Fig. 9). These processes, potentially linked to the recent demographic expansion of the parasite species evidenced by an increased abundance of infection in intermediate hosts (Levsen et al., 2022), may promote the occurrence of new haplotypes. In this regard, a demographic expansion of the cod population in recent years has been reported (Kjesbu et al., 2014; Rose and Rowe, 2015; ICES, 2021).

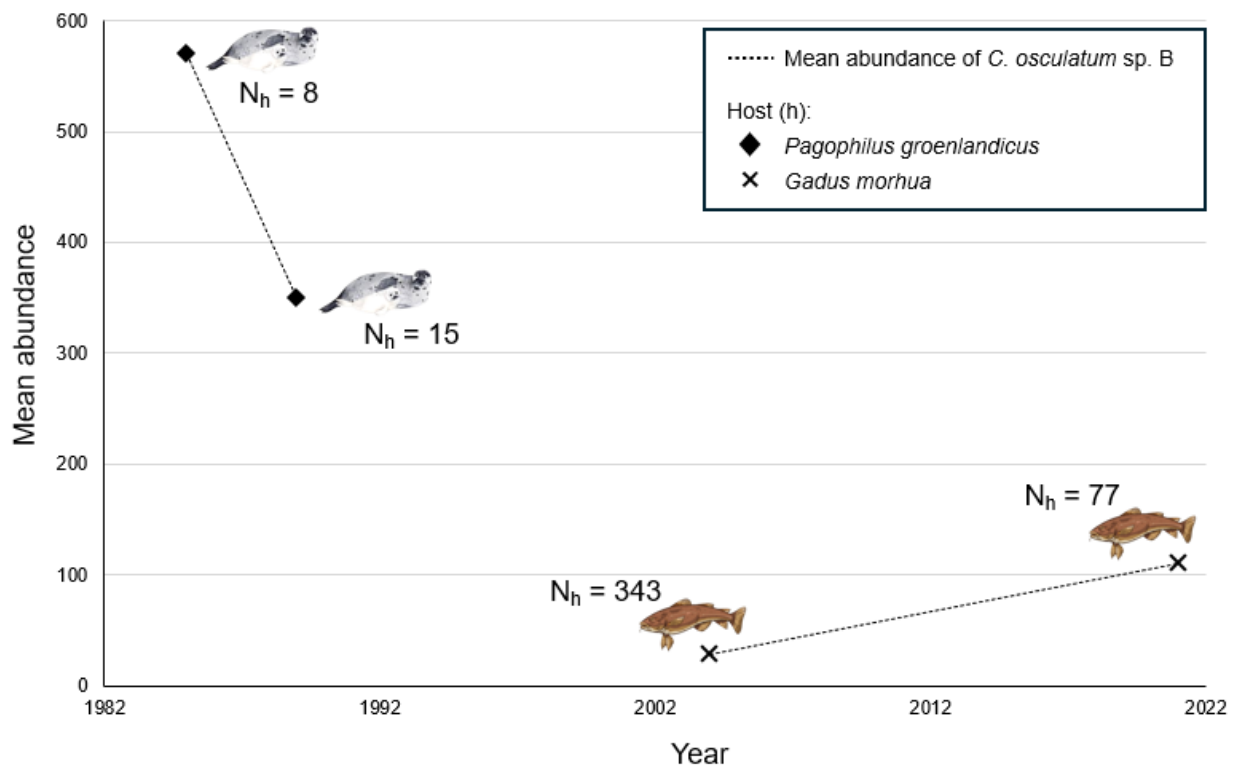


Figure 9. Mean abundance of *C. osculatum* sp. B in Arctic and sub-Arctic populations of *Pagophilus groenlandicus* (black diamonds) and *Gadus morhua* (crosses), showing demographic variations likely occurred in the past three decades, according to our repository data concerning seals infection, and records by Perdiguerro-Alonso et al. (2008) and Levsen et al. (2022) concerning fish infection.

Additionally, this may also be related to the potential immigration of *C. osculatum* sp. B, acquired by its hosts from other Arctic regions not investigated here, such as the Northwest Atlantic, where the species is also present (Nascetti et al., 1993). Such immigration could explain the occurrence of the new haplotypes detected in this study. Interestingly, in support of this hypothesis, some haplotypes identified in the present study in the contemporary populations were detected in recent analysis, from the Northwest Atlantic, specifically in Canadian waters (Severin et al., 2020) (GenBank accession numbers: MT448515 and MT448519). In contrast to the mitochondrial gene, analysis of the SSRs DNA loci showed a lower level of differentiation between the two temporal data sets (F_{st} close to zero). This may be due to the generally lower mutation rate of microsatellites compared to the mitochondrial DNA, as well as to their biparental inheritance, which can obscure the patterns of genetic differentiation observed in mtDNA.

A clear reduction in observed heterozygosity (H_o) over time and in the average number of alleles (A), was observed in the contemporary metapopulations of the parasite species within Pop 2. Moreover, the loss of rare alleles in the same contemporary samples, along with a lower frequency distribution of certain alleles (Fig. 7), provides further evidence of a decline in the parasite's

genetic variability. Rare alleles are often among the first to be lost during population bottlenecks (Gatto-Almeida et al., 2022). Taken together, these observations indicate possible genetic erosion events in the contemporary population sample, suggesting that *C. osculatum* sp. B populations examined in this study may have undergone a demographic decline over the past 35–40 years (Fig. 9).

The apparent contradiction between the observed decline in unique genetic variability in *C. osculatum* sp. B over the past 35–40 years, alongside the emergence of novel haplotypes, may be linked to fluctuations in host populations and subsequent changes in parasite abundance. Throughout this period, both seal and fish populations—critical hosts in the parasite’s life cycle—have undergone periods of decline due to viral outbreaks and overfishing, followed by recoveries driven by conservation efforts and fishery management. Given the parasite’s reliance on trophic transmission and high host specificity (Mattiucci & Nascetti, 2008), reductions in host abundance (Fig. 9), likely influenced its population size and genetic diversity.

Marine resource overexploitation and environmental shifts, including climate change, have contributed to habitat degradation and fluctuations in key fish stocks that serve as intermediate or paratenic hosts for *C. osculatum* sp. B, such as Arctic cod (*Gadus morhua*) and capelin (*Mallotus villosus*) (Bundy et al., 2009; Gjørseter et al., 2009). Similarly, pinniped populations—its definitive hosts—have experienced declines, including severe phocine distemper virus (PDV) epidemics that killed over 23,000 harbour seals in 1988 and 30,000 in 2002 (Härkönen et al., 2006), followed by substantial recoveries (Brasseur et al., 2018). Harp seals have also faced population fluctuations due to reduced seasonal sea ice, a crucial breeding platform (Johnston et al., 2012).

A comparable pattern has been observed in the Baltic Sea, where increasing abundance of *C. osculatum* (s. s.) in both cod and grey seals—its primary definitive hosts—coincide with a rapid expansion of seal populations (Haarder et al., 2014; Mehrdana et al., 2014; Nadolna & Podolska, 2014; Zuo et al., 2018). This recovery has likely contributed to the resurgence of parasite populations (Olsen et al., 2007), suggesting that shifts in host dynamics play a key role in shaping the genetic variability of *C. osculatum* sp. B.

The acquisition of new mitochondrial and nuclear variants in *C. osculatum* sp. B populations might align with reports of demographic expansion in Arctic seals and fish populations, as a consequence of conservation measures’ implementation. These measures may have facilitated the parasite’s demographic recovery after an initial decline, promoting the spread of novel haplotypes at the mtDNA *cox2* locus and new alleles at nuclear SSRs DNA loci. Therefore, the observed genetic changes in the parasite species likely reflect significant perturbations within the Arctic food web, driven by human-induced alterations.

However, the overall high level of heterozygosity across the SSRs DNA loci and the haplotype variation estimates indicates that these metapopulations of *C. osculatum* sp. B still maintain its high genetic diversity. This characteristic is crucial, as it may help natural populations to cope with and adapt to environmental changes (Birader, 2023).

The observations here acquired align with existing literature suggesting that climate-driven changes in Arctic marine ecosystems, such as rising sea temperatures influencing host distribution (Gerland et al., 2023), might also act as selective pressures on parasite populations. For example, warmer temperatures can directly affect the survival and transmission efficiency of parasite larval stages, while shifts in host migration patterns may influence the genetic composition of parasite populations (Davidson et al., 2011).

2.5 Conclusions

This study provides new insights into the population genetic structure of *C. osculatum* sp. B over a temporal scale spanning several decades, emphasizing the role of definitive seal and intermediate/paratenic fish host populations demography in shaping its genetic variability. The findings highlight how the genetic diversity and abundance of this trophically transmitted parasite can serve as a baseline for monitoring Arctic trophic webs.

Given that anthropogenic effects and environmental perturbations due to climate change are particularly evident in the Arctic ecosystem (Steiner et al., 2021; Deb and Bailey, 2023), this study underscores the value of a marine anisakid worm as an ecological indicator of how high-latitude host-parasite systems may respond to human impacts.

Future research should focus on integrating genetic data with biotic factors, such as additional host species, to explore different trophic interactions, as well as abiotic environmental variables, (ocean temperature rise, reduced sea-ice extent, changes in water chemistry). This will help clarify the relationship between anthropogenic stressors and parasite populations, leading to a better understanding of the ecological and evolutionary factors driving genetic diversity in the *C. osculatum* (s.l.) species complex.

Finally, investigating the host-parasite co-phylogeography between the *C. osculatum* (s.l.) species complex and their specific definitive hosts will provide valuable insights into the historical dynamics and microevolutionary events occurred in these parasites inhabiting Arctic-Boreal waters..

3. II Hybridization and introgression of mitochondrial genome between the two species *Anisakis pegreffii* and *A. simplex* (s. s.) using a wide genotyping approach: evolutionary and ecological implications.

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Abstract

Anisakis pegreffii and *A. simplex* (s.s.) are the two zoonotic anisakids infecting cetaceans, as well as pelagic demersal fish and squids. In European waters, *A. pegreffii* prevails in the Mediterranean Sea, while *A. simplex* (s.s.) prevails in NE Atlantic. Abiotic conditions likely play a significant role in shaping their geographical distribution. The Iberian Atlantic and Alboran Sea waters are sympatric areas of the two species, providing facilitating their reproductive interaction in cetacean hosts. A total of N= 429 adults and L3 stage larvae from both sympatric and allopatric areas were analyzed by a wide nuclear genotyping approach (including newly and previously found diagnostic SNPs at nDNA and microsatellite DNA loci), and sequenced at mtDNA *cox2*. Admixture between the two species was detected in the sympatric areas studied by STRUCTURE Bayesian analysis; NEWHYBRIDS revealed different categories of hybridization between the two species, representing approximately the 5%. A tendency for F1 female hybrids to interbreed with the parental species at the geographical distribution limits of both species, was observed. This finding suggests that hybridization occurs when the two parental species significantly differ in abundance. Mitochondrial introgression of *A. simplex* (s.s.) in *A. pegreffii* from Mediterranean waters was also detected, likely as a result of past and/or paleointrogression events. The high level of genetic differentiation between the two species, and versus their backcrosses indicates that, despite current hybridization, reproductive isolation which maintains evolutionary boundaries between the two species exists. Possible causes of hybridization phenomena are attempted, as well as their evolutionary and ecological implications, also considering a sea warming scenario in European waters.

Key words: *Anisakis*, DNA microsatellites, SNPs, mtDNA *cox2*, hybridization, mitochondrial introgression

3.1 Introduction

The evolutionary boundary between closely related parasite species represents an important window to study microevolutionary processes, speciation mechanisms, genetic architecture, and gene flow between interacting species. Such boundaries are often permeable, leading to natural hybridization phenomena in contact zones. Currently, attention is directed towards describing patterns of genetic variation across the genome of closely related parasites species to disentangle and characterise patterns of ongoing hybridization and introgression phenomena (Calvani and Slapeta, 2020).

Previous genetic, phylogenetic, morphological and ecological evidence supports the view that the two anisakid species of the *A. simplex* (s. l.) complex, i.e. *A. pegreffii* and *A. simplex* (s.s.), constitute two well-genetically differentiated sister taxa (Mattiucci *et al.* 2014). The two species differ in geographical distribution, phenotypic traits - such as differential *intra-vitam* (reviewed in Mattiucci *et al.* 2018) and *post-mortem* migration capability in the musculature of their intermediate/paratenic fish hosts (Cipriani *et al.* 2016; 2024) - as well as differential gene expression of some adaptive and immunomodulatory molecules under thermal cues (Palomba *et al.* 2019; 2020a). Certain environmental abiotic conditions, such as sea temperature (Gomes *et al.* 2023; Mladineo *et al.* 2023), represent determinant drivers in shaping their geographical ranges (Mattiucci *et al.*, 2018; Diez *et al.*, 2022; 2024). Similarly, biotic variables, such as host availability, diversity, and preference, also play a significant role (Mattiucci and Nascetti, 2008; Klimpel *et al.* 2010; Kuhn *et al.*, 2010; Shamsi and Barton 2023). Indeed, both species share a wide range of definitive hosts, mostly oceanic dolphins, mysticetes, and phocoenids (Mattiucci *et al.* 2018; Cipriani *et al.* 2022). The range of their intermediate/paratenic hosts includes a wide array of demersal and pelagic fish and squids, within the geographical distribution of the two species (reviewed in Mattiucci *et al.* 2018; Shamsi and Barton 2023). The two species exhibit allopatric zonation following a latitudinal gradient. *Anisakis pegreffii* is widespread in the Mediterranean Sea and the Austral region, between 30°S and 60°S; in the Atlantic, its northern distribution limit is the Iberian coast up to the Bay of Biscay (Diez *et al.* 2022; 2024). *Anisakis pegreffii* is also widely distributed in the south Pacific Ocean, from the East China Sea (Lee *et al.* 2009; Ding *et al.* 2022) to New Zealand waters, in sympatric condition with *A. berlandi* (Mattiucci *et al.* 2018; Bello *et al.* 2021; Shamsi and Barton 2023). Genetic sub-structuring of *A. pegreffii* from Austral and Boreal regions, as inferred from population genetics analyses using microsatellite loci (Mattiucci *et al.*, 2019), suggests that geographic distance restricts gene flow between ‘antipodean’ populations of this parasite species. ‘Despite the genetic sub-structuring, *Fst* values observed at five SSR loci indicate a high gene flow ($Nm = 7.8$) between Boreal and Austral

populations of *A. pegreffii* (Mattiucci *et al.*, 2019). This genetic pattern is likely shaped by different cetacean populations, oceanographic barriers, and the historical microevolutionary lineage diversification of the species in distinct waters (Mattiucci *et al.*, 2014; 2019; 2025; Blažeković *et al.*, 2015; Ding *et al.*, 2022).

Anisakis simplex (s.s.) distribution ranges approximately from 35°N to the Arctic Seas, circumpolarly. Its southern geographic limit in North-Eastern Atlantic waters seems to be the Iberian Atlantic coast (see Mattiucci *et al.* 2018). However, in some regions, the geographic ranges of the two *Anisakis* species (i.e. *A. pegreffii* and *A. simplex* (s.s.)) overlap. These regions include the NE Atlantic Ocean (Spanish-Portuguese coast) and the Western Pacific Ocean (East China Sea and Sea of Japan), where both species are often found in sympatry and syntopy, at both larval and adult stages (Abollo *et al.* 2001; Pontes *et al.* 2005; Lee *et al.* 2009; Suzuki *et al.* 2010; Chou *et al.* 2011; Quiazon *et al.* 2011; Mattiucci *et al.* 2016; Cipriani *et al.* 2022; Ding *et al.* 2022; Ramilo *et al.* 2023).

In these contact areas of their geographical distribution, the occurrence of "putative hybrids", or "recombinant genotypes" between *A. pegreffii* and *A. simplex* (s.s.) has been reported, either as larval stages, or as adults (Abollo *et al.* 2003; Martín-Sánchez *et al.* 2005; Umehara *et al.* 2006; Lee *et al.* 2009; Suzuki *et al.* 2010; Chou *et al.* 2011; Quiazon *et al.* 2011; Molina-Fernández *et al.* 2015; Abattouy *et al.* 2016; Gomez-Mateos *et al.* 2020), as well as from allopatric regions such as the Mediterranean Sea (Farjallah *et al.* 2008; Meloni *et al.* 2011; Chaligiannis *et al.* 2012; Cavallero *et al.* 2012, 2014; Pekmezci *et al.* 2014). However, these studies were typically based on a single marker (i.e. the ITS region of rDNA), lacking the resolution to determine whether the observed shared polymorphism between the two taxa was due to incomplete lineage sorting, historical introgression, ongoing hybridization, and/or introgression. Indeed, hybridization, introgression, and the retention of ancestral polymorphism in closely related parasitic taxa could generate patterns of genetic variation that complicate their disclosure, when choosing an unsuitable number of genetic/molecular markers (Anderson *et al.* 2001). Mattiucci *et al.* (2016) used a multilocus approach with nuclear markers (allozyme loci and EF1 α nDNA sequence analysis) to distinguish parental taxa and F1 hybrids among *A. pegreffii* and *A. simplex* (s.s.) specimens sampled along the Iberian Atlantic coast. Similarly, Roca-Gerones *et al.* (2021) detected F1 hybrids in the same sympatric area using the same markers. Both studies, however, reported discordant results when applying PCR–RFLP analysis of the ITS region of rDNA to the same specimens analyzed with the multilocus approach. This highlights the risk of misidentifying parental *Anisakis* species and their hybrid categories (F1 hybrids and backcrosses) when relying solely on a single nuclear marker. Given that a large number of diagnostic markers is required to accurately identify F1 and Fn hybrid categories (i.e., backcrosses) between closely related species (Tarroso *et al.*,

2014), a multilocus approach is essential for characterizing hybrid gene pools. True hybrid events in contact zones between closely related species involve amalgamations of independently evolved genomes, leading to intergenomic interactions. In these cases, evolutionary processes such as recombination and selection can occur, making it essential to study these phenomena using joint data from both nuclear and cytoplasmic markers. In this context, the combined use of some DNA microsatellite loci developed in species of the *A. simplex* (s.l.) complex (Mattiucci *et al.* 2019; Bello *et al.* 2020) and *Nas10* nDNA (Palomba *et al.* 2020b), has allowed to describe the first report on the natural interspecific hybridization between *A. pegreffii* and *A. berlandi* in one of their contact areas (Bello *et al.* 2021) included in the species' geographical ranges of distribution.

The use of microsatellite DNA loci has revealed the existence of sex-linked loci in the three species of the *A. simplex* (s.l.) complex (Mattiucci *et al.*, 2019; Bello *et al.*, 2021). These are genes located on the X-chromosome. These chromosomes are maternally inherited, as they lack a paternal chromosomal counterpart and thus possess only a single copy of the gene. In general, in ascaridoid nematodes males are XO, and females are XX (Müller and Tobler, 2000; Foster *et al.* 2020). However, exceptions are common, such as in *Ascaris suum*, which possesses 19 autosomes and five X chromosomes ($2n = 38A + 10X$ in females, $38A + 5X$ in males) (Müller and Tobler, 2000). Preliminary cytogenetic studies recently conducted *in vitro* on *A. pegreffii* have shown that this parasite species has a diploid chromosome number of $2n = 18A$ (Moratal *et al.*, 2023). However, no distinct chromosomal organization patterns between males and females were reported.

In the present paper, a multilocus genotyping approach was applied on several specimens of *A. pegreffii* and *A. simplex* (s.s.), incorporating a broader set of novel and previously detected diagnostic nuclear and mitochondrial loci. Additionally, a Bayesian population structure approach was applied and validated for the two species. The aims were to: *i*) to develop and validate new diagnostic nuclear markers between the two species; *ii*) define the genetic structure of the two species in the contact zone spanning the Atlantic Iberian coast and Mediterranean waters of the Alboran Sea, in comparison with their allopatric populations; *iii*) assess the occurrence of different patterns of ongoing hybridization between the two interacting species in this sympatric area; *iv*) detect mitochondrial introgression in their genome; *ivv*) investigate the causes of the distribution of these hybridization/introgression phenomena, as well the potential evolutionary and ecological implications of the findings; and, finally, *ivv*) disclose the potential occurrence of sex-linkage in the newly scored SSRs loci.

3.2 Materials and methods

3.2.1 Sample collection

A total of N = 429 *Anisakis* spp. specimens were collected from sampling localities in the North East Atlantic Ocean, Mediterranean Sea, and South Pacific waters (New Zealand) (Fig. 1). These locations include regions where the two sibling species *A. simplex* (s.s.) and *A. pegreffii* are reported both in allopatry (e.g., the North Sea for *A. simplex* (s.s.); the Central-Eastern Mediterranean Sea and New Zealand waters for *A. pegreffii*) and in sympatry (e.g., the Atlantic Iberian Peninsula and the Alboran Sea). Specifically, N= 314 third stage larvae (L3) were collected from various fish hosts, while N= 115 adult nematodes were obtained from cetacean host species. The adult specimens were first sex-discriminated according to the main morphological diagnostic features between males and females (Mattiucci *et al.* 2018). Details about sample collections, including the localities of the intermediate/paratenic (fish) and definitive (cetacean) hosts and the number of adult male and female specimens studied, are reported in Table 1. Collected nematodes were repeatedly washed in saline solution and preserved by freezing at -20°C in distilled water or stored in 70% alcohol until their genotyping.

Table 1. Number of specimens analysed from definitive and intermediate/paratenic hosts, reported with sampling area, genotyped at nuclear (*EF1 α -1*, *Nas10*, *Sod* and *Adk* nDNA and *SSRs* loci) and mitochondrial (mtDNA*cox2*) gene loci.

Sampling area	Cod e area	Host species	N	N _{A♂} ♂	N _{A♀} ♀	N _L 3	N <i>cox2</i> mtDNA	<i>EF1</i> α -1 nDNA	<i>Nas10</i> nDNA	<i>Sod</i> nDNA	<i>Adk</i> nDNA	<i>SSRs</i> DNA loci
Mediterranean Sea												
Adriatic Sea (42°18'N-15°35'E)	ADS	<i>Lophius piscatorius</i>	2	-	-	2	2	2	2	2	2	2
		<i>Merluccius merluccius</i>	2	-	-	2	2	2	2	2	2	2
		<i>Scomber scombrus</i>	3	-	-	3	3	3	3	3	3	3
		<i>Trachurus trachurus</i>	1	-	-	1	1	1	1	1	1	1
		<i>Stenella coeruleoalba</i>	18	3	15	-	18	18	18	18	18	18
		<i>Lepidopus caudatus</i>	21	-	-	21	21	21	21	21	21	21
Tyrrhenian Sea (41°7'N-13°24'E)	TYS	<i>Merluccius merluccius</i>	2	-	-	2	2	2	2	2	2	2
		<i>Scomber scombrus</i>	4	-	-	4	4	4	4	4	4	4
		<i>Merluccius merluccius</i>	10	-	-	10	102	102	102	102	102	102
Eastern Mediterranean Sea (39°93'N-16°69'E)	EMS	<i>merluccius</i>	2			2						
		<i>Phycis blennoides</i>	20	-	-	20	20	20	20	20	20	20
Torre Vieja (37°98'N-0°30'W)	TOR	<i>Merluccius merluccius</i>	2	-	-	2	2	2	2	2	2	2
Alboran Sea (35°59'N-3°41'W)	ALB	<i>Merluccius merluccius</i>	68	-	-	68	68	68	68	68	68	68
		<i>Trachurus trachurus</i>	3	-	-	3	3	3	3	3	3	3
NE Atlantic Ocean												
Gulf of Cádiz (36°19'N-6°40'W)	CDZ	<i>Merluccius merluccius</i>	16	-	-	16	16	16	16	16	16	16
Huelva (36°50'N-7°18'W)	HUE	<i>Merluccius merluccius</i>	18	-	-	18	18	18	18	18	18	18
Vigo (42°11'N-9°45'W)	VIG	<i>Merluccius merluccius</i>	6	-	-	6	6	6	6	6	6	6
		<i>Delphinus delphis</i>	30	11	19	-	30	30	30	30	30	30
		<i>Globicephala melas</i>	3	-	3	-	3	3	3	3	3	3
		<i>Phocoena phocoena</i>	9	6	3	-	9	9	9	9	9	9
Bay of Biscay (44°13'N-6°28'W)	BSB	<i>Micromesistius poutassou</i>	13	-	-	13	13	13	13	13	13	13
		<i>Trachurus trachurus</i>	1	-	-	1	1	1	1	1	1	1

continue

N_A ♂♂, number of adult male nematodes; N_A ♀♀, number of adult female nematodes; N_{L3}, number of species at third larval stage.

												continued
Scottish Sea (59°13'N- 0°14'W)	SCO	<i>Lagenorhynchus albirostris</i>	5	-	-	5	5	5	5	5	5	5
		<i>Stenella coeruleoalba</i>	5	-	5	-	5	5	5	5	5	5
Norwegian Sea (68°52'N- 3°08'E)	NWS	<i>Clupea harengus</i>	13	-	-	13	13	13	13	13	13	13
		<i>Micromesistius poutassou</i>	7	-	-	7	7	7	7	7	7	7
		<i>Scomber scombrus</i>	5	-	-	5	5	5	5	5	5	5
New Zealand (40°20'S- 172°37'E)	NZE	<i>Globicephala melas</i>	50	20	30	-	50	50	50	50	50	50
Tot:			429	40	75	314	429	429	429	429	429	429

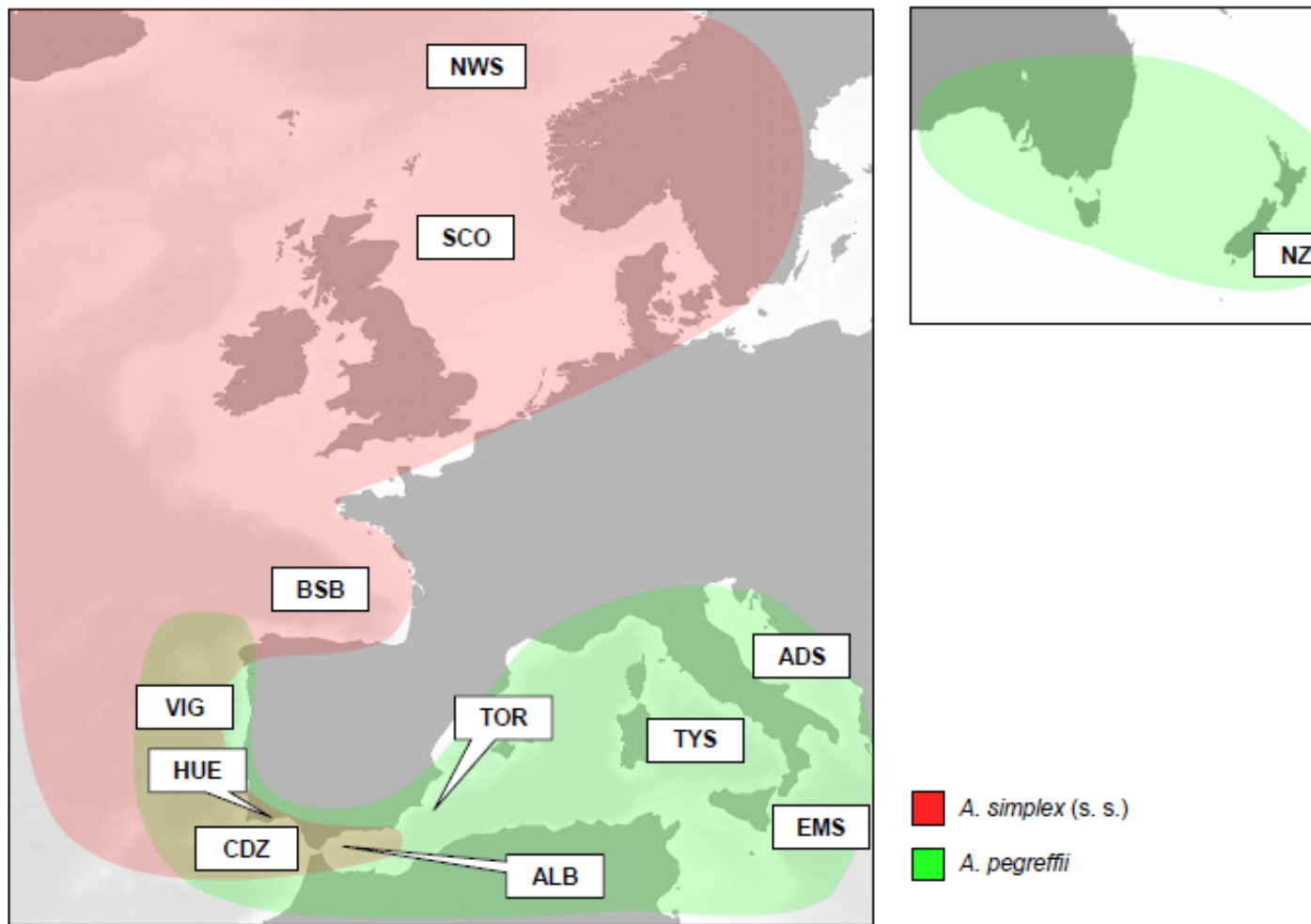


Figure 1. Collecting sites of *A. simplex* (s. s.) and *A. pegreffii* analyzed in the present study, mapped onto the geographical range of the two parasite species (colored in red and green, respectively). ADS: Adriatic Sea; TYR Tyrrhenian Sea; IOS: Ionian Sea; TOR: Torrevieja (Mediterranean Spain coast); ALB: Alboran Sea; CDZ: Cadiz (Atlantic Spain coast); HUE: Huelva (Atlantic Spain coast); VIG: Vigo (Atlantic Spain coast); BSB: Bay of Biscay; SCO: Seas west of Scotland; NWS: Norwegian Sea; NZE: New Zealand coast

3.2.2 Multi-markers genotyping approach based on nuclear and mitochondrial genes

Approximately 2 mg of tissue was cut from each larval and adult specimen, using a portion from the cephalic end for adults. DNA extraction was performed using the Quick-gDNA Miniprep Kit (ZYMO RESEARCH), according to the manufacturer's protocol. The 429 sampled individuals were first genotyped using nuclear markers (*EF1 α -I* and *Nas10* nDNA gene loci) previously developed for the *A. simplex* (s.l.) complex. PCR amplification of the elongation factor *EF1 α* nDNA was performed using the primers EF-F and EF-R (Table 2) following the procedures detailed in Mattiucci *et al.* (2016). For the *Nas10* nDNA gene, PCR amplification was performed using the primers *Nas10-F* and *Nas10-R* (Table 2) following the procedures detailed in Palomba *et al.* (2020b).

The same specimens were then genotyped for a total of 7 microsatellite loci (i.e., *Anisl 00314*, *Anisl 00875*, *Anisl 00185*, *Anisl 05784*, *Anisl 07132*, *Anisl 08059*, *Anisl 10535*), previously developed and validated in the three species of the *A. simplex* (s.l.) complex (Mattiucci *et al.* 2019; Bello *et al.* 2020). Additionally, other 4 SSR loci (i.e. *Anisl 4*, *Anisl 7*, *Anisl 15*, and *Anisl 22*) among those developed in Mladineo *et al.* (2017a) were here scored for the first time on specimens of the two parasite species. The amplification of microsatellite loci was carried out in three multiplex PCR reactions: *Anisl 05784*, *Anisl 08059*, *Anisl 00875* and *Anisl 07132* in Multiplex 1; *Anisl 00185*, *Anisl 00314*, *Anisl 10535* and *Anisl 7* in Multiplex 2; *Anisl 4*, *Anisl 15*, and *Anisl 22* in Multiplex 3 (Table 2). The PCR conditions followed those described in Mattiucci *et al.* (2019). To increase the number of diagnostic loci necessary to discriminate patterns of hybridization between the two species, additional nuclear gene loci, i.e. the superoxide dismutase (*Sod*) and the adenylate kinase (*Adk*) nDNA, were selected. Primers for these loci were designed for this work based on transcriptome sequences of *A. pegreffii* previously deposited by Palomba *et al.* (2022) using the online software program Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>). PCR amplifications were performed using the primers SOD-F and SOD-R for the *Sod* nDNA gene and ADK-F and ADK-R for the *Adk* nDNA gene (see Table 2). Both PCRs were carried out in a 25 μ L volume containing 1 μ L of each 10 mM primer, 2 μ L of 25 mM MgCl₂ (Promega), 5 μ L of 5 $\times$ buffer (Promega), 0,8 μ L of 10 mM mix containing the four dNTPs (Promega), 5 U of Go-Taq Polymerase (Promega) and 2 μ L of total DNA. The PCR conditions for amplification of *Sod* nDNA were: 95 °C for 2 min (initial denaturation), followed by 40 cycles at 95 °C for 30 s (denaturation), 57 °C for 45 s (annealing), 72 °C for 90 s (extension) and followed by post-amplification at 72 °C for 7 min. The PCR conditions for amplification of *Adk* nDNA were: 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for 1 min. The two new gene loci were thus

validated on the same specimens (N= 431) of *A. pegreffii* and *A. simplex* (s. s.) that had previously been analyzed using other nuclear markers. The purified PCR products from all markers were sequenced by an external Company (Bio-Fab Research©).

Finally, all the specimens tested at those nuclear loci were sequenced at the mitochondrial *cox2* gene (Mattiucci *et al.* 2014) using the primers 211F and 210R (Nadler and Hudspeth 1998) (Table 2), following the procedures detailed in Mattiucci *et al.* (2014).

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Table 2. Locus name, primers sequences, and fragment bp size of the gene loci used in the present study. For SSRs nuclear loci, the repeat type and the fluorescent dye used to label the primer are also reported.

Primer sequence 5' – 3'	Size (bp)	NSNP		
F: TTTTCTAGTTATATAGATTG R: CACCAACTCTTAAAATTATC	600			
F: TCCTCAAGCGTTGTTATCTGTT R: AGTTTGGCCACTAGCGGTTCC	409	2		
F: GAGAGTCATTCTCGC R: TCATGTATTTCCGAC	451	1		
F: AGAAGGTGAGCCAACGACTG R: GGCATTTCCCGTCTTCAAGC	706	6		
F: AACAGGCCGTCTCTTCCATC R: ACGAGGACTGCAGCTGAATC	1062	8		
	Size range (bp)	Repeat type	Dye	N _A
F: GGGTTTGGACACTGGTTTGG* R: TGCAATCGTCATTTCTGCCTC	69 – 105	(TGT) 11	VIC	16
F: CCCTTCTCTCTGTGGAGTCG* R: TGCTGCTATTCGAGCGTTTG	98 – 126	(CATC) 4	PET	13
F: TGACGCTCGAGTTGGTACAG* R: GGTGGTGATGTTTACGCGAC	142 – 172	(GCA) 8	NED	11
F: ATCAGTGCCGAGTAGCATGG* R: TTCAGGGTGCAAATGACGTG	208 – 252	(ATTG) 7	FAM	12
F: CGTAGTGCTTCGCTTATCGC* R: AGGGGATATGATCGAGATTAGACAG	96 – 132	(GATA) 7	VIC	10
F: GTTTTGGGTTACCAACGACC* R: GCAATGGGCAGTCATGGAAG	125 – 146	(TTG) 9	PET	8
F: CCGTGAACGCGATTCTCAAC* R: CCGCCTCCAAACAAACAAAC	185 – 209	(TTG) 7	FAM	9
F: TGATTGCGATTACTATTATTGTCAGT* R: GGTGGTGTCGTGACAGTGAT	216 – 279	(TAT)8(TTA)5ctatttattattttaatattatcat(TTA)8tc(ATT)6	NED	18

F: ACAAGGAATGCTCGTCGATATGA*	130 – 193	(TAA) 9	FAM	25
R: TCACTCTTGTCCAGCACTGC				
F: GCAGACACTCATTCGCAGTC*	231 – 251	(CAAT) 7	NED	6
R: GCTGATCCCTTGAAGTGGCT				
F: TCAGAGGTGCGTAACGTTCA*	185 – 263	(TTA) 30	PET	24
R: GCCACATCCATCCAAAAGAGC				

* = labelled primer. Fluorescent dyes: VIC = green; PET = red; NED = yellow, FAM = blue.

3.2.3 Genetic data analysis

The sequences obtained from the N= 429 individuals at both mitochondrial and nuclear gene loci were edited and then aligned with reference sequences using the software ClustalX v.2. The same sequences were also analysed using Genemapper v.4.1 and PeakScanner v.2. (Applied Biosystems, Waltham, MA, USA). MICRO-CHECKER v.2.2.3 (Van Oosterhout *et al.* 2004) was used to check for genotyping errors. ARLEQUIN v.3.5 (Excoffier and Lischer 2010) was used to calculate the genetic diversity indices observed at the 11 SSRs loci, i.e., the observed heterozygosity (Ho), the expected heterozygosity (He), and the mean number of alleles per locus (A). This software also assessed the occurrence of Hardy-Weinberg Equilibrium (HWE) at each SSR locus through exact test outcomes, and it was also used to estimate the fixation index FIS from the genetic data sets acquired from the SSRs loci in the analyzed populations of the two *Anisakis* species. In addition, AMOVA and matrix of pairwise genetic differences (FST) (Wright 1949, 1965) were calculated using ARLEQUIN v.3.5 on microsatellite and nuclear SNPs data.

A model-based Bayesian clustering algorithm was implemented in STRUCTURE v.2.3.3 (Pritchard *et al.* 2000) using the genotypes at the diagnostic nuclear SNPs and diagnostic and partially diagnostic (between the 95% and 99%) SSRs loci, in order to evaluate the admixture of pure lineages and to estimate mixing proportions between *A. simplex* (s.s.) and *A. pegreffii* in the analyzed samples.

With the number of clusters (K) set between 1 and 3, twenty replicates of the analysis were carried out, each with 100,000 MCMC iterations and a burn-in period of 50,000 steps. The admixture model assumes that each individual has ancestry from one or more of K genetically distinct sources (Lawson *et al.* 2018). The best K value was identified using both the log probability of the data and the rate of change in the log probability of the data between successive K values as the optimality criteria (Evanno *et al.* 2005). A model-based Bayesian clustering method implemented in NEWHYBRIDS v.1.1 (Anderson and Thompson, 2002) was used to evaluate the most parsimonious allocation of samples. Specifically, the analysis tested for the presence of pure parental individuals (*A. simplex* (s.s.) or *A. pegreffii*), F1 hybrids and backcrossed individuals to both species. TCS haplotype network construction was carried out using PopART v.1.7 (Clement *et al.* 2002). The analysis was performed using the statistical parsimony procedure (95% parsimony connection limit), implemented in TCS v.1.21 (Templeton *et al.* 1992).

3.3 Results

3.3.1 Genotyping *Anisakis* spp. specimens by EF1 α -1 and Nas 10 nDNA regions

Sequences of 409 bp from the nuclear EF1 α -1 nDNA partial region were obtained for all 429 larval and adult specimens analyzed in this study (Table 1). This fragment revealed two diagnostic SNPs distinguishing *A. pegreffii* from *A. simplex* (s.s.), as reported by Mattiucci *et al.* (2016). Based on this analysis, N = 340 specimens, homozygous for these SNPs, were assigned to *A. pegreffii*, whereas N = 70 specimens homozygous for the alternative allele were assigned to *A. simplex* (s. s.). Heterozygous genotypes at these diagnostic loci were found in N= 19 individuals (Fig. 2) from the sympatric areas here studied. A total of six sequences of the EF1 α -1 nDNA region were deposited in GenBank under the accession numbers XXXXXXXX for *A. pegreffii*, and XXXXXXXX for *A. simplex* (s. s.).

Similarly, a 451 bp sequence fragment of the Nas10 nDNA region was obtained for all 429 specimens analyzed. The sequences revealed one diagnostic SNP between *A. simplex* (s. s.) and *A. pegreffii* (see Palomba *et al.* 2020b). This position resulted homozygous CC in the *A. simplex* (s. s.) individuals (N = 70) from both its sympatric and allopatric populations; whereas, it was always homozygous GG in *A. pegreffii* (N = 338) from its allopatric and sympatric populations here examined. However, patterns of heterozygote genotypes at that diagnostic position were found in those individuals N= 19 also resulted heterozygous at the EF1 α -1 nDNA as above described, plus 2 specimens; all of them collected from the sympatric areas here studied (Fig. 2).

A total of six sequences of the Nas10 nDNA region were deposited in GenBank under the following accession numbers XXXXXXXX for *A. pegreffii*, and XXXXXXXX for *A. simplex* (s. s.).

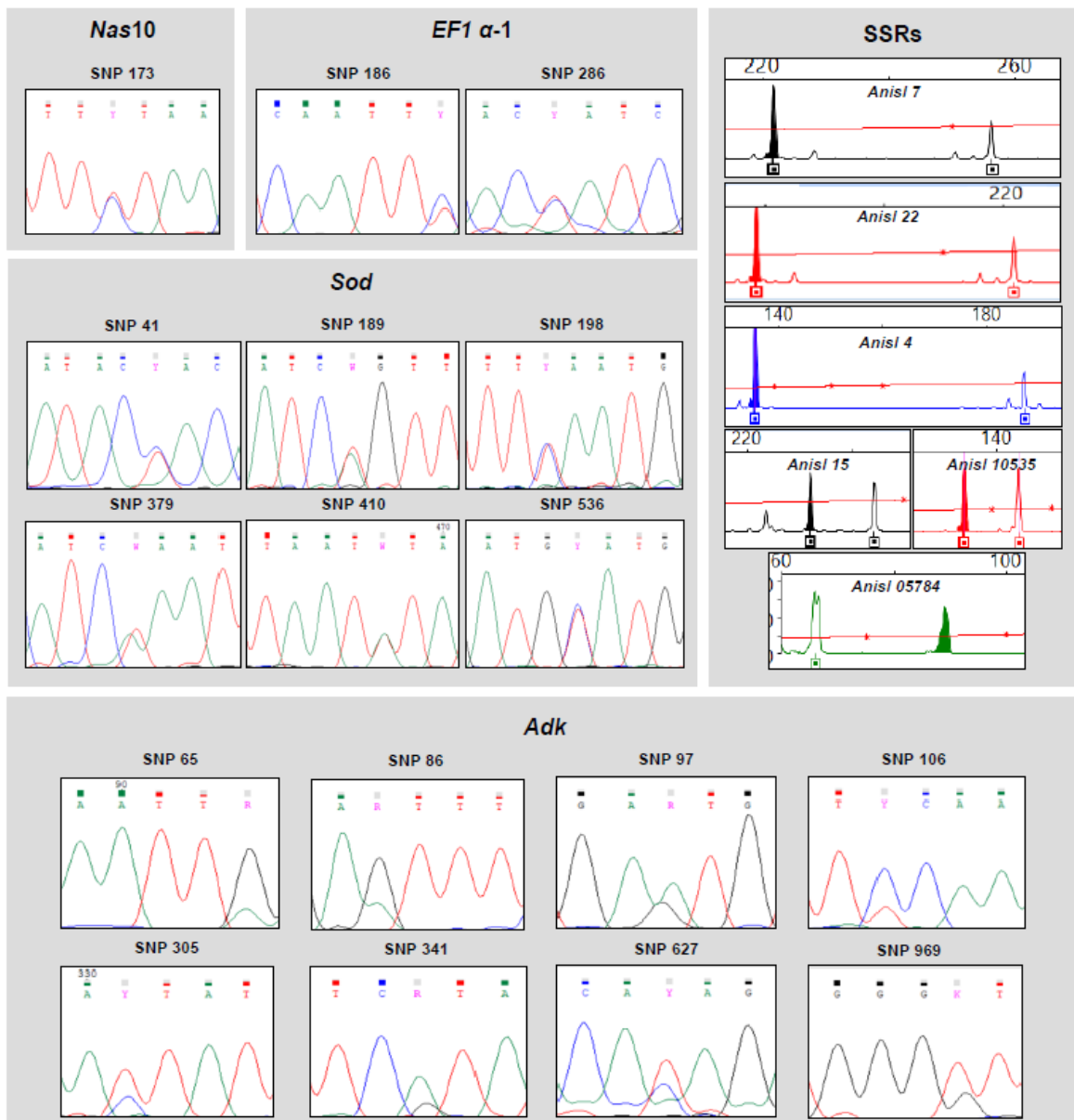


Figure 2. Chromatograms at the diagnostic SNPs in *Nas10*, *EF-1 α -1*, *Sod* and *Adk* nDNA loci and electropherograms of the 5 diagnostic and partially diagnostic SSRs loci (*Anisl 7*, *Anisl 22*, *Anisl 15*, *Anisl 4* and *Anisl 10535*), showing the heterozygous genotype having a double peak between *A. simplex* (s. s.) and *A. pegreffii* exhibited by admixed specimens between the two species.

3.3.2 Diagnostic SNPs at the novel *Sod* and *Adk* nDNA gene loci

Fixed diagnostic nucleotide positions capable of discriminating between the two species were detected at both *Sod* and *Adk* nDNA gene loci (Fig. 3a and Fig. 3b). In particular, a 706 bp fragment of the *Sod* nDNA region was obtained for all of the 429 specimens analysed. This fragment revealed the presence of 6 fixed alternative (i.e diagnostic) SNPs between *A. pegreffii* and *A. simplex* (s. s.) (Fig. 3a). Based on these positions, N= 340 specimens, being homozygous for the 6 diagnostic SNPs, were assigned to *A. pegreffii*, whereas N = 78 specimens also homozygous for the alternative SNPs, were assigned to *A. simplex* (s. s.). Finally, a total of N = 11 specimens showed a heterozygous genotype at all six diagnostic positions (shown in Fig. 2). These individuals were among the N= 21 also observed as heterozygous at the diagnostic SNPs found at the *Nas10* nDNA. A total of six sequences of the *Sod* nDNA region were deposited in GenBank under the accession numbers XXXXXX for *A. pegreffii*, and XXXXXX for *A. simplex* (s. s.). Similarly, a 1062 bp fragment of the *Adk* nDNA region was obtained from the same N = 429 allopatric and sympatric specimens of the two targeted species. This fragment revealed the presence of 8 diagnostic SNPs between *A. pegreffii* and *A. simplex* (s. s.), showing fixed alternative nucleotides between the two species (Fig. 3b). Based on these diagnostic positions, N = 338 specimens were assigned to *A. pegreffii*, whereas N = 71 specimens, showing the opposite SNPs were assigned to *A. simplex* (s. s.). Among the total number of tested specimens, N= 20 individuals showed a heterozygous genotype at all the eight diagnostic SNPs (shown in Fig. 2) among the among the N= 21 heterozygous found at the diagnostic SNPs found at the *Nas10* nDNA. A total of six sequences of the *Adk* nDNA region were deposited in GenBank under the accession numbers XXXXXX for *A. pegreffii*, and XXXXXX for *A. simplex* (s. s.).

3.3.3 Novel diagnostic SSRs DNA loci

Finally, the same 429 individual specimens were also genotyped at 11 scored SSR DNA loci within the metapopulations of *A. pegreffii* and *A. simplex* (s.s.) (Table 3a). Differences in the overall genetic diversity values of the different populations of the two species were observed, based on both the mean number of alleles per locus, and the values of expected heterozygosity per locus (H_e) (Table 3a). All the microsatellite loci scored in the present study were polymorphic, with the total number of alleles varying in *A. pegreffii* between 5 (e.g., at loci *Anisl* 7 and *Anisl* 22) and up to 13 at the newly scored locus *Anisl* 4 (Table 3a). In *A. pegreffii*, the mean number of alleles per locus (A) across the 11 SSRs loci was 8.7. In *A. simplex* (s.s.), the mean number of alleles per locus (A) was 9.6 with the newly scored locus *Anisl* 22 showing the highest number of alleles $N=25$; while the minimum number of alleles was found at the loci *Anisl* 10535 and *Anisl* 8059 (Table 3a).

No significant deviations between observed (H_o) and expected (H_e) heterozygosity were observed at loci *Anisl* 10185 and *Anisl* 08059 in all analysed samples of *A. pegreffii*. Similarly, in *A. simplex* (s. s.), no -significant departures from HWE were observed at loci *Anisl* 10535, *Anisl* 07132, *Anisl* 05784 and *Anisl* 08059 (Table 3a). Significant deviations from HWE were found in both species at loci *Anisl* 00185 and *Anisl* 7, as well as at the newly tested loci *Anisl* 4, *Anisl* 22 and *Anisl* 15 (Table 3a). These loci showed a marked excess of homozygotes in the two species (Table 3a), with positive F_{IS} values (Suppl. Fig.1a). Generally, positive F_{IS} values indicate an excess of homozygote genotypes at the selected loci; while negative values indicate an excess of heterozygotes from the expected HWE (Suppl. Fig.1a). When dividing the genotypes at these five loci into adult male and female worms, they were found to be in HWE in the female genotypes (Table 3b). The male individuals of both species were homozygous at those loci ($F_{IS} = 1$) (Suppl. Fig.1b), likely due to their hemizyosity state, indicative of X-linked inheritance. At the newly scored locus *Anisl* 15, the specimens of *A. pegreffii* were monomorphic (Table 3b), showing only the allele 231 (Suppl. Table 1), preventing the demonstration of sex-linkage in *A. pegreffii*. In contrast, this locus was sex linked in *A. simplex* (s.s.), as were the other two newly tested SSR loci, i.e. *Anisl* 4, *Anisl* 22 in both species. Accordingly, no significant departures from the HWE were found between the expected homozygous and heterozygous females at these loci, nor at locus *Anisl* 7 in the two *Anisakis* spp. (Table 3b). Analogously, no significant departure from HWE was found at the locus in any of the populations of *A. pegreffii* (Table 3b).

Table 3a. Genetic diversity, at 11 SSRs loci, in populations of *A. pegreffii* and *A. simplex* (s. s.) analysed in the present study. No estimates were possible for the locus *Anisl 15* because it was monomorphic in all the *A. pegreffii* populations.

Locus	<i>A. pegreffii</i>		<i>A. simplex</i> (s. s.)
<i>Anisl 00185</i>	N	157	61
	H_O	0.69	0.68
	H_E	0.78	0.72
	<i>p value</i>	0.03	0.16
	A	8	7
<i>Anisl 00314</i>	N	158	62
	H_O	0.39	0.19
	H_E	0.77	0.79
	<i>p value</i>	***	***
	A	7	7
<i>Anisl 10535</i>	N	158	62
	H_O	0.34	0.12
	H_E	0.33	0.16
	<i>p value</i>	0.79	0.03
	A	7	5
<i>Anisl 07132</i>	N	158	63
	H_O	0.71	0.53
	H_E	0.75	0.73
	<i>p value</i>	0.39	0.02
	A	10	9
<i>Anisl 05784</i>	N	158	61
	H_O	0.81	0.57
	H_E	0.78	0.73
	<i>p value</i>	0.08	0.02
	A	11	10
<i>Anisl 08059</i>	N	157	61
	H_O	0.78	0.37
	H_E	0.85	0.41
	<i>p value</i>	0.04	0.02
	A	13	5
<i>Anisl 00875</i>	N	158	61
	H_O	0.43	0.55
	H_E	0.44	0.70
	<i>p value</i>	0.18	0.06
	A	8	9
<i>Anisl 7</i>	N	155	50
	H_O	0.20	0.46
	H_E	0.63	0.67
	<i>p value</i>	***	***
	A	5	8
<i>Anisl 4</i>	N	154	60
	H_O	0.39	0.56
	H_E	0.73	0.91
	<i>p value</i>	***	***
	A	13	15

continue

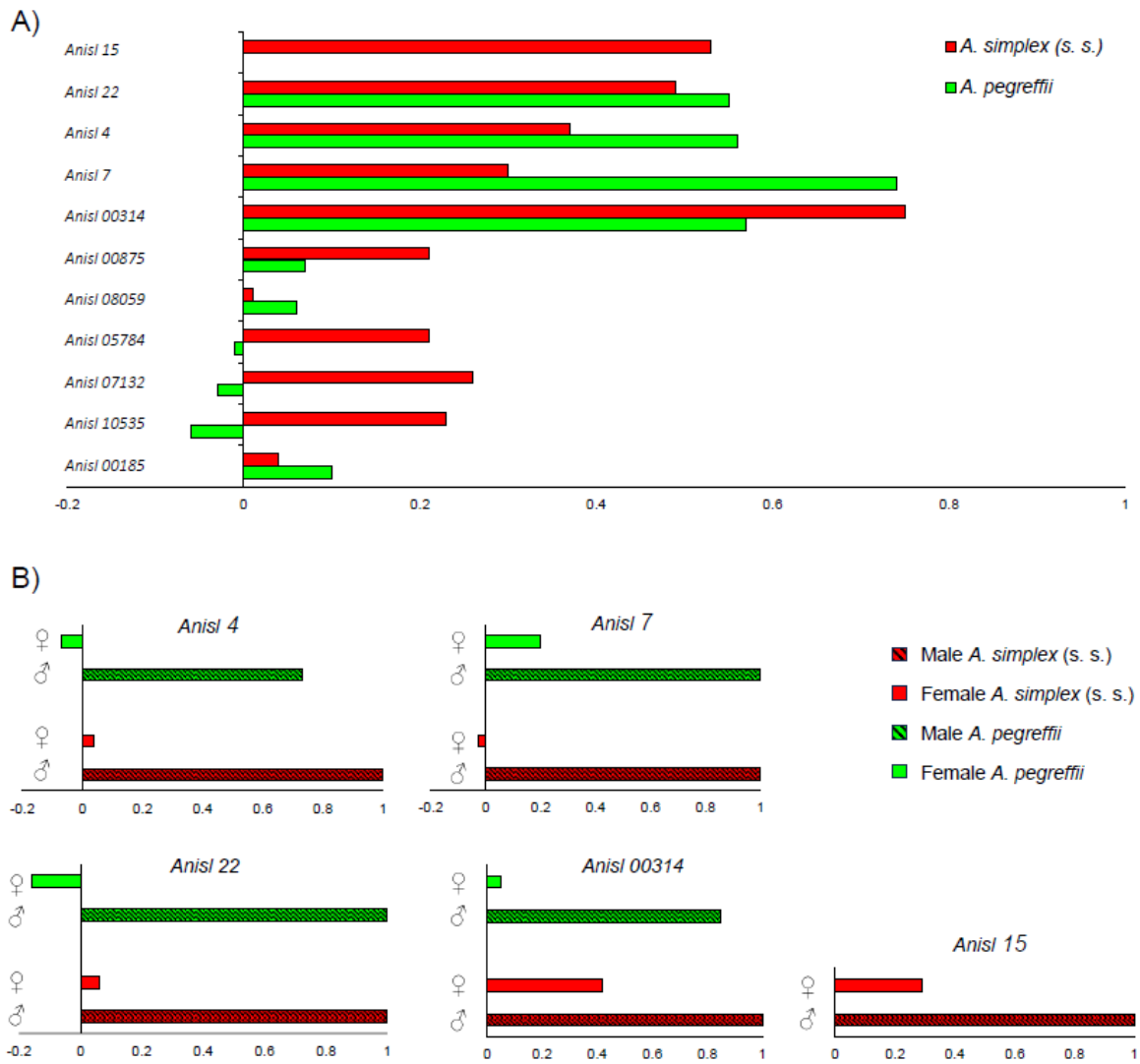
continued			
<i>Anisl 4</i>	N	154	60
	H_O	0.39	0.56
	H_E	0.73	0.91
	p value	***	***
	A	13	15
<i>Anisl 22</i>	N	155	63
	H_O	0.32	0.47
	H_E	0.61	0.93
	p value	***	***
	A	5	25
<i>Anisl 15</i>	N	-	62
	H_O	-	0.40
	H_E	-	0.70
	p value	-	***
	A	-	6

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 value, significance ($p < 0.05$) of the deviation from HWE expectation. *** $p < 0.01$.

Table 3b. Genetic diversity at the five sex-linked loci (i.e. *Anisl 00314*, *Anisl 7*, *Anisl 4*, *Anisl 22*, and *Anisl 15*) in *A. pegreffii* and *A. simplex* (s. s.) estimated in adult female specimens. Estimates at the locus *Anisl 15* were not possible because it is monomorphic in *A. pegreffii*.

Locus		<i>A. pegreffii</i>	<i>A. simplex</i> (s. s.)
		♀♀	♀♀
<i>Anisl 00314</i>	N	28	16
	H_O	0.75	0.43
	H_E	0.79	0.75
	p value	0.67	0.03
<i>Anisl 7</i>	N	28	16
	H_O	0.42	0.75
	H_E	0.53	0.72
	p value	0.37	0.39
<i>Anisl 4</i>	N	28	17
	H_O	0.75	0.88
	H_E	0.70	0.92
	p value	0.99	0.52
<i>Anisl 22</i>	N	28	17
	H_O	0.60	0.88
	H_E	0.52	0.93
	p value	0.34	0.26
<i>Anisl 15</i>	N	-	17
	H_O	-	0.52
	H_E	-	0.74
	p value	-	0.02

N: number of genotyped specimens at each locus; H_O : observed heterozygosity; H_E : expected heterozygosity; p value: significance ($p < 0.05$) of the deviation from HWE expectation.



Supplementary Figure 1. a) F_{IS} value calculated at eleven SSRs DNA loci studied in the two species *A. pegreffii* and *A. simplex* (s. s.), (except *Anisl 15* because of the monomorphism observed in *A. pegreffii*). **b)** F_{IS} value in male and female adult specimens at the sex-linked loci in *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.

Allele frequencies estimated in *A. pegreffii* and *A. simplex* (s.s.) are reported in Suppl. Table 1. For the sex-linked loci (i.e., *Anisl* 00314, *Anisl* 7, *Anisl* 4, *Anisl* 15 and *Anisl* 22) allele frequencies were calculated considering the hemizyosity of male specimens, with males considered monoallelic and females biallelic, according to Mattiucci *et al.* (2019). Among the newly tested SSR loci, *Anisl* 4 and *Anisl* 15 and *Anisl* 22 showed significant differences in allele frequencies between the two sibling species (Fig. 4). Indeed, *Anisl* 15 and the *Anisl* 7 were 100% diagnostic between the two species. A total of N = 11 specimens showed at these SSRs loci both the allele of the parental taxon *A. simplex* (s.s.) and that of *A. pegreffii* (Fig. 2).

Supplementary Table 1. Allele frequencies, observed at eleven microsatellite loci, in populations of *A. pegreffii* and *A. simplex* (s. s.) here studied. As regards the sex-linked loci (*), the most reliable estimate of allele frequencies was calculated according to the sex-linked genetic model estimate, assuming: (i) the hemizyosity of the males at that locus in the two *Anisakis* species; (ii) their adult female counterparts, as biallelic at the sex-linked loci.

Locus	Alleles	<i>A. pegreffii</i>	<i>A. simplex</i> (s. s.)
<i>Anisl</i> 00185	188	0.01	0.04
	191	0.11	0.04
	194	0.27	0.47
	197	0.30	0.25
	200	0.20	0.14
	203	0.09	0.03
	206	0.02	0.03
<i>Anisl</i> 00314	96	0.04	0.10
	100	0.25	0.25
	104	0.29	0.35
	108	0.26	0.17
	112	0.10	0.09
	116	0.04	-
	120	0.02	0.02
<i>Anisl</i> 10535	125	-	0.01
	131	0.02	0.03
	134	0.02	0.91
	137	0.03	0.05
	140	0.81	0.01
	143	0.10	-
	146	0.01	-
<i>Anisl</i> 07132	208	-	0.03
	212	0.05	0.03
	216	0.16	0.02
	220	0.42	0.41
	224	0.13	0.31
	228	0.13	0.08
	232	0.03	0.09
			continue
			continued
	236	0.05	0.03

	236	0.05	0.03
	240	0.02	-
	248	0.01	-
<i>Anisl 05784</i>	63	-	0.24
	66	-	0.08
	69	-	0.04
	72	-	0.01
	75	-	0.02
	78	0.01	0.45
	81	0.06	0.12
	84	0.07	0.03
	87	0.20	0.01
	90	0.35	0.01
	93	0.18	-
	96	0.06	-
	99	0.04	-
	102	0.01	-
	105	-	-
<i>Anisl 08059</i>	82	0.01	0.03
	86	0.28	0.90
	90	0.04	0.05
	94	0.11	-
	98	0.15	0.01
	102	0.10	0.01
	106	0.11	-
	110	0.06	-
	114	0.09	-
	118	0.03	-
	122	0.01	-
	126	0.01	-
<i>Anisl 00875</i>	142	-	0.01
	145	-	-
	148	0.01	-
	151	0.04	0.02
	154	0.06	0.05
	157	0.72	0.27
	160	0.13	0.44
	163	0.03	0.18
	166	-	0.02
	169	-	0.01
<i>Anisl 7</i>	216	0.16	-
	219	0.50	-
	222	0.31	-
	225	0.03	-
	249	-	0.03
	252	-	0.15
	255	-	0.49
	258	-	0.21
	261	-	0.09
	264	-	0.01
	267	-	0.01
	270	-	0.01
			continue
			continued
<i>Anisl 4</i>	130	0.01	-
	133	0.35	-

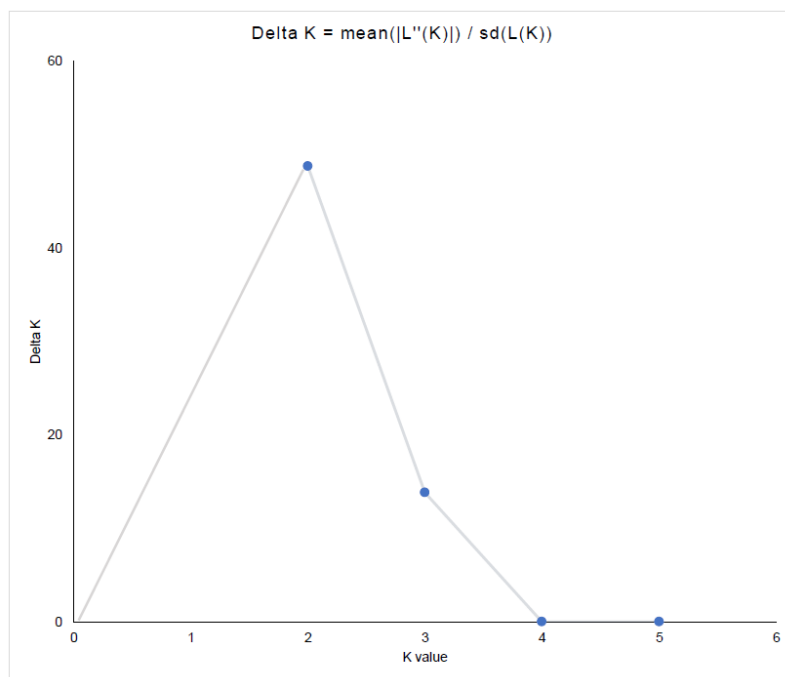
	136	0.25	-
	139	0.27	-
	142	0.05	-
	145	0.02	-
	148	0.02	-
	151	0.01	-
	157	-	0.02
	160	0.01	0.01
	163	-	0.03
	166	0.01	0.03
	169	-	0.07
	172	-	0.15
	175	-	0.14
	178	-	0.09
	181	-	0.09
	184	-	0.12
	187	-	0.14
	190	-	0.06
	193	-	0.06
	199	-	0.01
<i>Anisl 22</i>	182	0.01	-
	185	0.40	-
	188	0.46	0.01
	191	0.11	0.02
	194	0.01	-
	200	-	0.10
	203	-	0.02
	206	-	0.02
	209	-	0.03
	212	-	0.06
	215	-	0.06
	218	-	0.18
	221	-	0.04
	224	-	0.08
	227	-	0.06
	230	-	0.05
	233	-	0.05
	236	-	0.07
	239	-	0.02
	242	-	0.01
	245	-	0.05
	248	-	0.04
	251	-	0.01
	260	-	0.02
	263	-	0.01
	266	-	0.01
	272	-	0.01
<i>Anisl 15</i>	231	1.00	0.01
	235	-	0.08
	239	-	0.28
	243	-	0.38
	247	-	0.22
	251	-	0.03



Figure 4. Distribution of allele frequencies at the partially diagnostic and 100% diagnostic SSRs loci between *A. pegreffii* (green) and *A. simplex* (s.s.) (red).

3.3.4 Identification of genetic admixture between the two species by Bayesian multi-markers genotyping approach

The results inferred from the Bayesian clustering algorithm of all 429 genotyped specimens performed by STRUCTURE 2.3.3., using the N = 17 100% diagnostic SNPs found in the nuclear loci, *plus* the genotypes found at the partially diagnostic loci (between 95% and 99%) SSRs loci between the two parental species, i.e. *Anisl 10535* and *Anisl 05784*, of the 429 specimens, are presented in Fig. 5a. We excluded sex-linked loci (i.e., *Anisl 15*, *Anisl 7*), despite being 100% diagnostic between the two parental taxa, in order to include in the analysis also the male specimens. Using the K = 2 clustering option, implemented by the Evanno method, the data set best fit a $\Delta K = 48.765$.



Supplementary Figure 2. Estimate of the best number of clusters (K) describing the population structure of the *Anisakis* spp. samples analysed in the present study. The computation was carried out by using the DeltaK optimality criteria.

According to the STRUCTURE analysis, it was possible to assign with a posterior probability of assignment of 100%, N= 406 specimens to the two pure parental taxa: *A. pegreffii* or *A. simplex* (s.s.). In particular, N= 337 individuals were identified as *A. pegreffii*, including both adults and L3 of *Anisakis* (Fig. 5a; Table 4). Similarly, N = 69 individuals were assigned to *A. simplex* (s.s.) with a posterior probability of $p= 100\%$ (Fig. 5a; Table 4). Finally, a total of N = 23 individuals,

as evidenced by the results obtained by the single nuclear marker above reported, showed admixtures in their nuclear genome, including L3 and adult specimens collected from the Atlantic Iberian and Alboran Sea coast (Fig. 5a; Table 4). In the case of these admixed individuals, NEWHYBRIDS, carried out on the same genotypes observed at all the nuclear gene loci tested, disclosed that N = 11 individuals were F1 hybrids between the two parental taxa, with 100% posterior probability (Fig. 5b). Additionally, NEWHYBRIDS showed that among the remaining 12 individuals showing admixture genome in the STRUCTURE analysis, N= 3 individuals were backcrosses to *A. pegreffii*, while N= 9 were backcrosses to *A. simplex* (s.s.) (Fig. 5b; Table 4). Only one adult female resulted to be F1 hybrid genotype; while all the backcrossed individuals were L3 stage larvae collected from fish hosts (Table 4). Concordant results between STRUCTURE and NEWHYBRIDS were achieved in the assignment of the remaining 406 specimens tested to the two pure parental genomes, *A. pegreffii* or *A. simplex* (s.s.), with 100% posterior probability (Table 4).

Table 4. Number of specimens assigned to different classes of introgression or to parental species: Pure parental *A. pegreffii*; Pure parental *A. simplex* (s.s.); F1 Hybrid; Backcross x *A. pegreffii*; Backcross x *A. simplex* (s. s.), on the basis of a Bayesian clustering approach implemented in STRUCTURE and NEWHYBRIDS combining the diagnostic SNPs at nDNA gene loci and SSRs loci. The number of

Area	Host species	Multinuclear genotyping assignment					mtDNA <i>cox2</i>	
		Pure parental <i>A. pegreffii</i>	Pure parental <i>A. simplex</i> (s. s.)	F1 Hybrid	Backcross <i>A. pegreffii</i>	Backcross <i>A. simplex</i> (s. s.)	<i>A. pegreffii</i>	<i>A. simplex</i> (s. s.)
ADS	<i>Lophius piscatorius</i>	2	-	-	-	-	2	-
	<i>Merluccius merluccius</i>	2	-	-	-	-	-	2
	<i>Scomber scombrus</i>	3	-	-	-	-	3	-
	<i>Trachurus trachurus</i>	1	-	-	-	-	1	-
	<i>Stenella coeruleoalba</i>	18	-	-	-	-	18	-
TYS	<i>Lepidopus caudatus</i>	21	-	-	-	-	21	-
	<i>Merluccius merluccius</i>	2	-	-	-	-	-	2
	<i>Scomber scombrus</i>	4	-	-	-	-	4	-
EMS	<i>Merluccius merluccius</i>	102	-	-	-	-	96	6
	<i>Phycis blennoides</i>	20	-	-	-	-	14	6
TOR	<i>Merluccius merluccius</i>	1	1	-	-	-	1	1
ALB	<i>Merluccius merluccius</i>	60	5	1	1	1	61	7
	<i>Trachurus trachurus</i>	3	-	-	-	-	3	-
CDZ	<i>Merluccius merluccius</i>	12	2	-	1	1	13	3
HUE	<i>Merluccius merluccius</i>	15	1	2	-	-	15	3
VIG	<i>Merluccius merluccius</i>	-	1	1	1	3	1	5
	<i>Delphinus delphis</i>	19	11	-	-	-	19	11
	<i>Globicephala melas</i>	-	3	-	-	-	-	3
	<i>Phocoena phocoena</i>	-	7	1	-	1	2	7
BSB	<i>Micromesistius poutassou</i>	2	3	5	-	3	4	9
	<i>Trachurus trachurus</i>	-	-	1	-	-	-	1

continue

specimens by mtDNA *cox2* gene locus is also represented.

continued								
SCO	<i>Lagenorhynchus</i>	-	5	-	-	-	-	5
	<i>albirostris</i>							
	<i>Stenella</i>	-	5	-	-	-	-	5
	<i>coeruleoalba</i>							
NWS	<i>Clupea harengus</i>	-	13	-	-	-	-	13
	<i>Micromesistius</i>	-	7	-	-	-	-	7
	<i>poutassou</i>							
	<i>Scomber scombrus</i>	-	5	-	-	-	-	5
NZE	<i>Globicephala melas</i>	50	-	-	-	-	50	-
Tot:		337	69	11	3	9	328	101

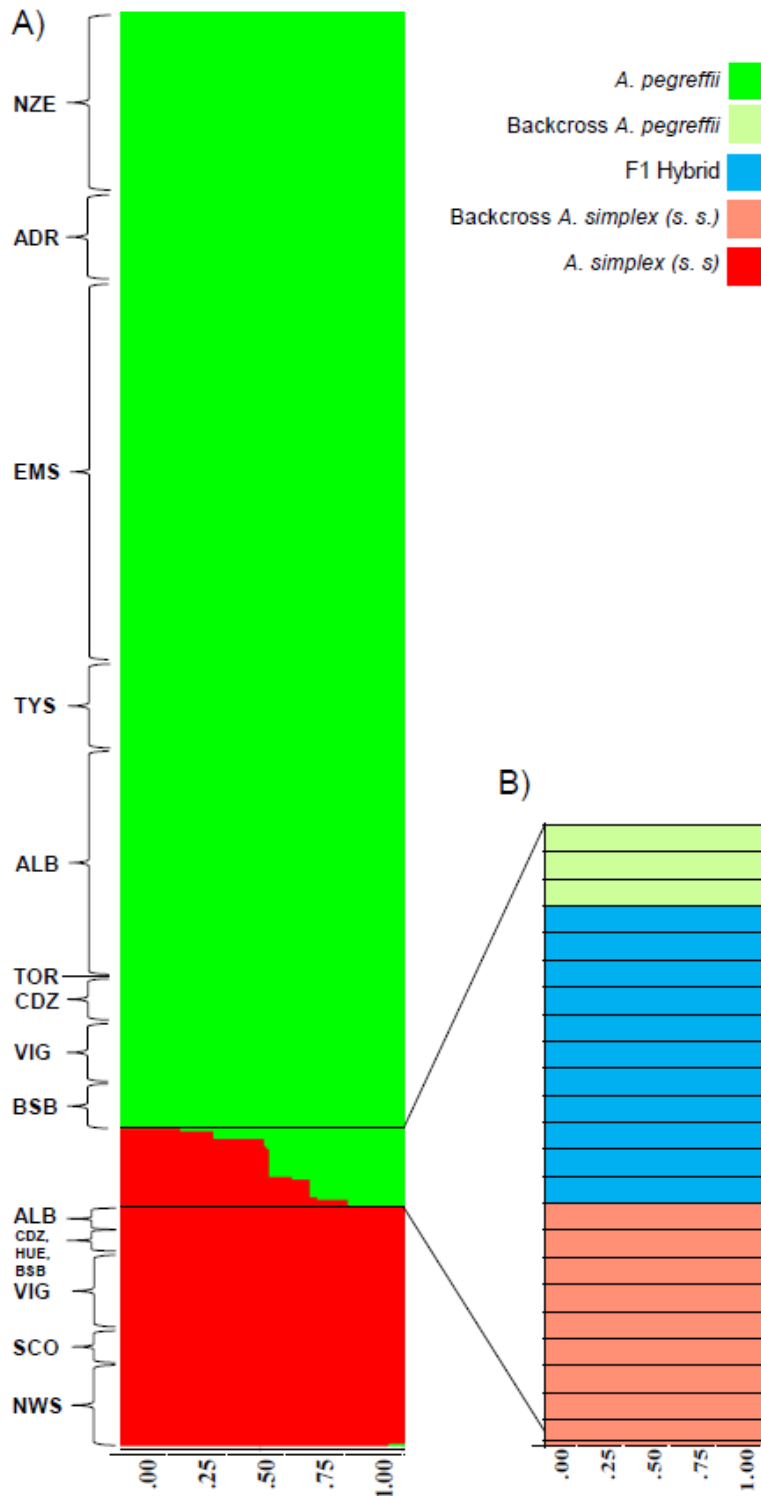


Figure 5. **A** Percentage contribution (Q) of each specimen of *A. pegreffii* and *A. simplex* (s. s.) to each cluster following a Bayesian clustering analysis implemented in STRUCTURE on 17 diagnostic SNPs at nDNA gene loci (*EF1 α -1*, *Nas10*, *Sod*, *Adk*) and at the SSRs loci e.i.e. *Anisl 10535* and *Anisl 05784*) (sex-linked were excluded to include male specimens in the elaboration), with K=2. **B.** Posterior probability of assignment to one of five genotype classes following a Bayesian clustering analysis implemented in NEWHYBRIDS. Those specimens with evidence of admixture were assigned to pure *A. pegreffii* and *A. simplex* (s. s.), F1 Hybrids, or backcrossed categories with 100% posterior probability value of assignment.

3.3.5 Mitochondrial inference of the pure and hybrid specimens

The results obtained from the mtDNA *cox2* sequences analysis of all those N = 429 specimens studied at the nuclear gene loci, are reported in Table 4. The haplotype parsimony network (TCS) inferred from mtDNA *cox2* sequences of all the specimens, as recognized by the STRUCTURE and NEWHYBRIDS analyses, showed the presence of two major haplogroups represented by the two pure parental taxa (Fig. 6). The TCS analysis of mtDNA *cox2* haplotypes resulted in a star-like tree for both haplogroups (Fig. 6). The haplotypes H23, H26, H30 and H31 were found as the most frequent in the pure parental specimens of *A. pegreffii* recognized by the STRUCTURE analysis of the nuclear genome. Analogously, specimens showing the nuclear genome of *A. simplex* (s.s.) recognized by STRUCTURE inference, mostly exhibited the haplotypes, H7 and H14 shared by all metapopulations of the parasite species (Fig. 6). Full concordance with nuclear genotyping was obtained in the recognition of all pure specimens collected from the allopatric areas, i.e. of *A. pegreffii* from off New Zealand coast and of *A. simplex* (s.s.) collected from the Norwegian Sea. The TCS network also revealed that among the confirmed F1 hybrids detected by NEWHYBRIDS, N = 4 had *A. pegreffii* maternal inheritance (Fig 6 and Table 4), whereas N = 7 had *A. simplex* (s.s.) maternal inheritance (Fig. 6 and Table 4). The haplotypes shown by these F1 individuals were also observed in the respective parental taxa: H1, H2, H3, H4, H5, H7, H8 in *A. simplex* (s.s) and H6, H9, H21 in *A. pegreffii*. Additionally, 1 backcross of *A. pegreffii* had *A. pegreffii* maternal inheritance, showing the haplotype H9. Other larval backcrosses to *A. pegreffii* found in *M. merluccius* from the Alboran Sea and Cadiz had *A. simplex* (s.s.) maternal inheritance showing haplotypes H15 and H22, which were shared with other pure specimens of *A. simplex* (s.s.). Most backcrosses of *A. simplex* (s.s.), observed as L3 in fish from NE Atlantic waters had *A. simplex* (s.s.) maternal inheritance, showing haplotypes H7, H10, H11, H12, H13, H14, while 2 backcrosses of *A. simplex* (s.s.) had *A. pegreffii* maternal inheritance showing haplotypes H16 and H17.

Interestingly, among the N= 337 individuals assigned to *A. pegreffii* with 100% posterior probability value by STRUCTURE and NEW HYBRIDS, N = 16 specimens collected from Adriatic, Ionian and Tyrrhenian Sea, showed a mito-nuclear discordance. Indeed, the mtDNA *cox2* of these specimens belonged to the *A. simplex* (s.s.) haplogroup (Fig. 6). In particular, 4 individuals shared the same H7 haplotype previously observed in other *A. simplex* (s.s.) specimens from Atlantic Iberian coast (Murphy *et al.* 2009; Klapper *et al.* 2016; Pons-Bordas *et al.* 2020; Simsek *et al.* 2020; Moratal *et al.* 2023) and Adriatic Sea waters (Buselic *et al.* 2015). The remaining individuals (N = 12) exhibited the haplotypes H18, H19, H20, H21 and H22 (Fig. 6). Among these haplotypes, H20 was previously observed in the Mediterranean Sea waters (Buselic *et al.* 2015),

and their sequences matched (100%) with those obtained from the Adriatic Sea and Tunisian coast (GenBank Accession number XXXXXX). Whereas, the H18, H19, H21 and H22 resulted as new haplotypes, not matching with other sequences deposited in GenBank (Fig. 6).

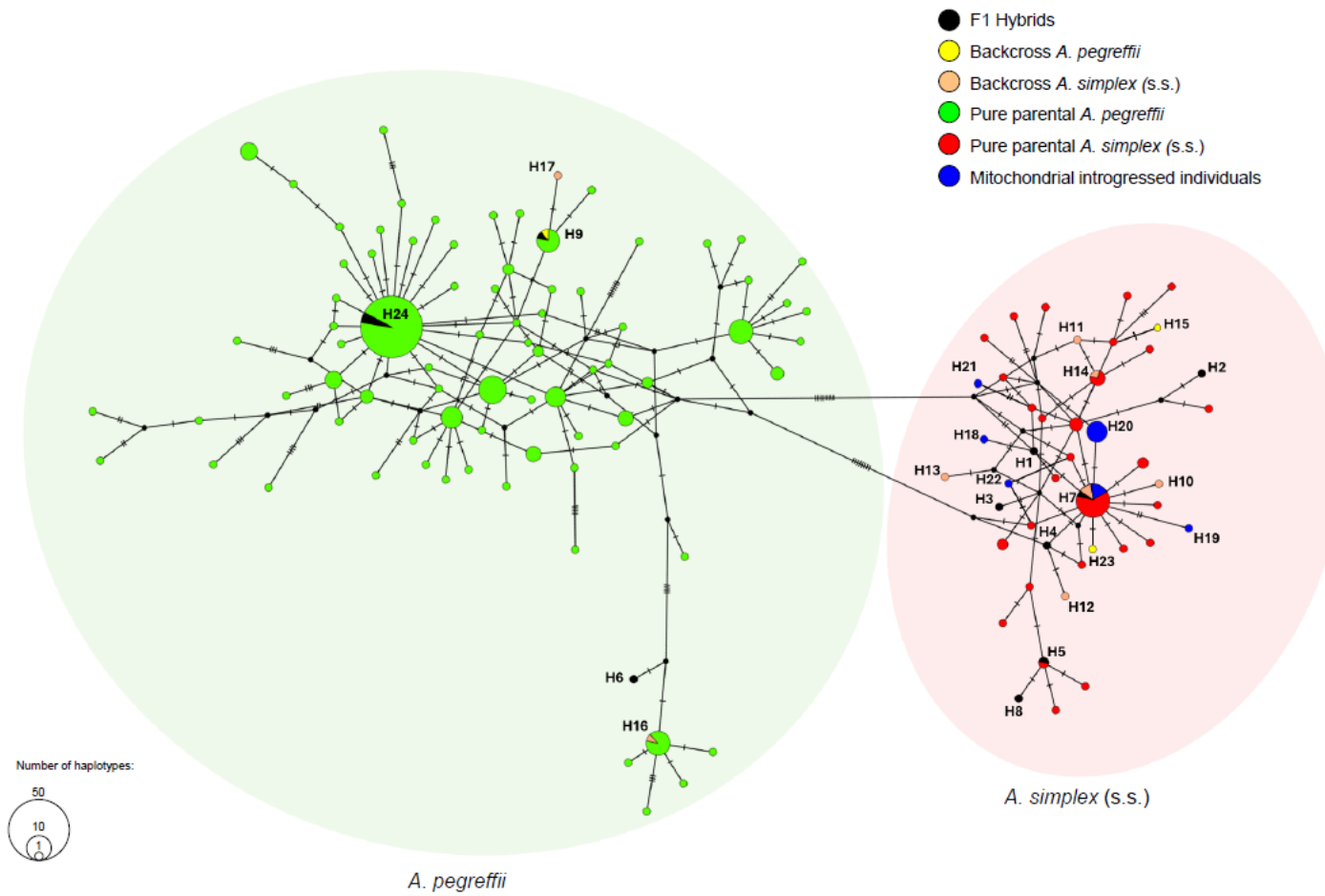


Figure 6 Statistical parsimony haplotype network among mitochondrial *cox2* locus haplotypes showed by pure specimens of the two parental species *A. pegreffii*, *A. simplex* (s.s.) and by their introgressed individuals, *plus* the specimens from Adriatic, Ionian and Tyrrhenian Sea waters showing mitochondrial introgression. Circles suggest the two different haplogroups represented by the parental species (*A. pegreffii*: light green, *A. simplex* (s.s.): light red).

3.3.6 Interspecific and intraspecific genetic differentiation inferred from nuclear and mitochondrial data

Excluding the sex-linked loci due to male hemizyosity, the AMOVA analysis of all nuclear gene loci analyzed in this study revealed that, for both species, most of the genetic variation was significantly allocated within individuals ($\approx 72\%$ and $\approx 95\%$). Notable variation was found among individuals within populations, with $FIT = 0.27$ ($p = 0.0000$) in *A. pegreffii* and $FIT = 0.05$ ($p = 0.3206$) in *A. simplex* (s.s.) (data not shown). A high level of genetic differentiation was found at the interspecific level between the metapopulations of *A. pegreffii* and *A. simplex* (s.s.), based on the SSRs loci and the SNPs at the nuclear DNA scored, with an average $Fst \approx 0.95$. A significant genetic differentiation was also observed between backcrosses to *A. simplex* (s.s.) vs pure parental *A. pegreffii* (on average, $Fst \approx 0.64$), as well as between backcrosses to *A. pegreffii* vs pure parental *A. simplex* (s.s.) (on average, $Fst \approx 0.60$) (data not shown). Lower but significant differentiation values were observed between F1 hybrids and the parental taxa ($Fst \approx 0.45$ and $Fst \approx 0.49$, respectively, versus *A. pegreffii* and *A. simplex* (s.s.)). Similarly, at the cytoplasmic level, a significantly high level of differentiation ($Fst = 0.73$; $p < 0.0000$) was inferred from the mtDNA *cox2* data set, between the two haplogroups (i.e. *A. pegreffii* and *A. simplex* s.s.).

3.4 Discussion

3.4.1 Novel nuclear diagnostic markers between *A. pegreffii* and *A. simplex* (s.s.)

Efforts have been recently made to pinpoint genome regions and individual SNPs that are diagnostic (i.e 100% alternative) between the three sibling species in the *A. simplex* (s.l.) complex (i.e. *A. simplex* s.s., *A. pegreffii* and *A. berlandi*), to study their geographical contact zones (Mattiucci *et al.* 2016; 2019; Gomez-Mateos *et al.* 2020; Bello *et al.* 2021). For the first time, we identified and validated genetic diagnostic variants in novel autosomal nuclear DNA of *A. simplex* (s.s.) and *A. pegreffii*, using a large number of specimens from both allopatric populations and the contact zones along the Atlantic Iberian coast and Mediterranean Sea waters. Indeed, 14 fixed diagnostic alternative SNPs were discovered in two newly analyzed autosomal gene loci, i.e. *Sod* and *Adk* nDNA (Fig. 3a and b). In addition, further SSRs loci, including autosomal and sex-linked ones, among those developed and studied in *A. simplex* (s.l.) (Mattiucci *et al.* 2019; Bello *et al.* 2020), were found to be of diagnostic value for distinguishing the two parental taxa here studied. In particular, the loci *Anisl* 4, *Anisl* 7, *Anisl* 22 and *Anisl* 15 were found to have 99% or 100% of diagnostic value between the two taxa (Fig. 4). Similarly, among these loci, *Anisl* 15 showed also fixed alternative alleles between *A. berlandi* and *A. pegreffii* (Bello *et al.* 2021). Finally, *Anisl*

10535 and *Anisl* 05784 have been confirmed as partially diagnostic between the two taxa, as previously observed (Mattiucci *et al.* 2019).

3.4.2 Novel sex-linked SSRs loci

The validation of the newly scored SSRs loci *Anisl* 4, *Anisl* 22, and *Anisl* 15 in a large sample of *A. simplex* (s.s.) individuals (N = 69) revealed the existence of additional sex-linked microsatellite loci in this species (i.e. *Anisl* 00314 and *Anisl* 7) (Mattiucci *et al.* 2019). Indeed, while loci *Anisl* 4 and *Anisl* 22 were previously found to be sex-linked in *A. pegreffii* (Bello *et al.* 2021), the same loci, along the *Anisl* 15, were identified for the first time as sex-linked in *A. simplex* (s.s.) in this study.

Sex linked loci are genes located on the X-chromosome. In our sample, which included both male and female adult specimens, we verified the potential occurrence of sex-linkage in the newly scored SSRs loci. They inherit the chromosome(s) from the mother only, lacking the paternal chromosomal counterpart, thus possessing just one copy of that gene. So far, the proportion of sex-linked microsatellites in the whole genome of the *A. pegreffii* and *A. simplex* (s.s.) can be estimated at about 33%, as found in our previous SSRs analysis in *A. berlandi* (Bello *et al.* 2021). XX/XO female/male sex determination appears to be common among nematodes (Harvey and Viney, 2001), since it is frequently reported that males have one less chromosome than females (Walton, 1940). To date, preliminary cytogenetic studies recently carried out on *A. pegreffii* (Moratal *et al.*, 2023) have revealed that *A. pegreffii* seems to possess a diploid number of $2n = 18$, likely consisting of autosomal holocentric chromosomes (Moratal *et al.* 2023). However, no specific data on sexual chromosomes were provided by the same Authors. Additionally, by using a telomeric probe (TTAGGC) $_n$ in *A. pegreffii*, Moratal *et al.* (2023) identified the presence of a conserved telomeric sequence, with variability in signal intensity at the telomeric ends of chromosomes. Hypervariability in the length of telomeric repeats is not unusual in animals (Rocco *et al.*, 2001), likely due to the low copy number of DNA repeats and the non-clustered organization of the tandem repeats (Moratal *et al.*, 2023). It has been also hypothesised that the low number of chromosomes found in some cells of *A. pegreffii* is due to the loss of chromosomes during the metaphase (Moratal *et al.*, 2023). However, no data concerning this phenomenon was provided by the same Authors. Further cytological work, combined with short and long-read sequencing technology, would shed light on whole chromosome-assemblage genomes, including sex chromosomes of the anisakid parasites, and their evolutionary significance.

In addition, the possible use of sex-linked loci for female/male identification has been recently discussed (Bello *et al.* 2021), and the finding of the new sex-linked loci, here achieved, will offer

in future studies a higher resolution power for their use in distinguishing male and female worms of these parasites, at any life-history stage.

3.4.3 Distribution of different patterns of nuclear and mitochondrial introgression

According to the biological species concept (Mayr, 1963; Arnold, 1997), which defines species as reproductively isolated, interspecific hybrids should be extremely rare. In reality, the evolutionary boundaries between species are permeable, with some estimates suggesting that hybridization may occur in up to 10% of animal species, and 25% in plants (Schwenk *et al.* 2008). The ubiquity of natural hybridization in terrestrial environments suggests it as a major driving force in evolutionary biology (Abbott 2013), even if it can also lead to evolutionary dead-ends and genetic swamping if hybrids are rare and isolated (Porretta and Canestrelli 2023). Previously, naturally occurring hybrids among parasites were thought to be quite rare, but NGS analyses have recently enabled the detection of different patterns of natural hybridization in closely related parasites species (Criscione *et al.* 2007; Steinauer *et al.* 2008; Huyse *et al.* 2009; Chaudhry *et al.* 2015; Hecht *et al.*, 2016; Leger and Webster, 2017; Calvani and Slapeta, 2020), as well as in parasites' vectors (Weetman *et al.* 2014; Mancini *et al.* 2015; Caputo *et al.* 2021). Most of these studies have been carried out in contact zones, where the biogeographic distribution of these parasites or vectors species, overlaps. The two *Anisakis* species here studied in their contact zone are genetically well differentiated, as evidenced by the high *Fst* values observed at both nuclear and mitochondrial levels. This implies that multiple markers are required to characterize their hybrids' gene pools. With multiple diagnostic nuclear markers, the probability of misclassification of an individual into an alternative category is very low and can be estimated by Mendelian principles. Indeed, when a backcrossing event is hypothesized to occur with the same parental species, several diagnostic nuclear markers may allow the identification of hybrid origin up to the third generation of backcrossing (fourth generational hybrids). Thus, the multilocus assay used in the present study, employing a wider set of diagnostic SNPs ($N = 17$) was able to capture patterns of admixture reflection between the parental genomes. In fact, the NEWHYBRIDS analysis clearly shows that interspecific hybridization between the two species has led to genetic introgression of parental lineages (Fig. 5b). The presence of first- and later-generation of hybrids among specimens of *A. pegreffii* and *A. simplex* (s.s.) from the contact zone of Atlantic Iberian coast and Alboran Sea indicates that recent introgression has occurred, demonstrating contemporary interspecific gene exchange. The combined analysis of microsatellites and SNP loci indicate symmetric patterns of genomic hybridization, with approximately 5% of admixture between the two interacting species. It would be also interesting to test by a multigenotyping approach, whether gene introgression between *A. pegreffii* and *A. simplex* (s.s.) exhibits similar rates in other sympatric areas of their

distribution (Mattiucci *et al.*, 2018; Ding *et al.*, 2022), potentially reflecting the species' evolutionary history.

To date, introgression has not been reported between the two more phylogenetically divergent taxa of the *A. simplex* (s.l.) complex, *A. pegreffii* and *A. berlandi*. Indeed, only F1 hybrids, but not later generations of hybrid individuals, have been found between them in their overlapping geographical range in New Zealand waters (Bello *et al.*, 2021).

3.4.4 Ecological and evolutionary implications of the hybridization and mitochondrial introgression

In parasites, hybridization and introgression could have microevolutionary and ecological implications, including potential emerging public health concern (Webster *et al.* 2013; Huyse *et al.* 2013; Bossier *et al.* 2015; Chaudhry *et al.* 2015). In our samples, the presence of hybrids appears to be driven by both ecological and evolutionary factors.

The achieved results suggest that there is a strong tendency for hybridization to take place between the two parental species when they greatly differ in abundance, and a tendency of the F1 hybrids to cross with the parental females at the respective limits of the geographical distribution of the two species. Although conclusions are only tentative, because the number of hybrid categories discovered is small with respect to the parental specimens, the data suggest a density-dependent mating pattern. The scarcity of the rarer species along the latitude distribution of the two species may be an important factor in increasing the likelihood of interspecific hybridization. Indeed, numerical disparity between adult specimens of *A. pegreffii* and *A. simplex* (s.s.) has been generally observed in the contact zone here studied (Levsen *et al.* 2018; Cipriani *et al.* 2022) along a latitudinal gradient, co-infecting the same individual definitive hosts.. The ecological conditions required by the two species shift in this geographic area, initially are favoring *A. pegreffii* and, moving northward, *A. simplex* (s.s.). Stochastic processes related to demographic disparities between the two interacting species at the limit of their respective geographic distribution in NE Atlantic waters – specifically, the Bay of Biscay for *A. pegreffii*, (Diez *et al.* 2022; 2024), and conversely, the Morocco Atlantic coast for the species *A. simplex* (s.s.), where this species has been rarely detected at its larval stage (Mattiucci *et al.* 2018) - could occur.

The numerosity of the two species in their hosts follows this pattern. Under these circumstances, the syntopic disparity of the two species occurring at adult stage in a definitive host from a contact area, may promote heterospecific mating pairings. Indeed, among the F1 and backcrosses individuals found at the northern part of the sympatric area here studied, most of them have shown a mitochondrial genome of *A. pegreffii*, indicating that they were originated from females at the geographic limit of the of the parasite species distribution (Diez *et al.* 2022; 2024) (Fig. 7b). The

opposite trend was observed in the southern part of the sympatric area (i.e. Portuguese and Alboran Sea coast), from where most of the F1 and backcrosses individuals have shown *A. simplex* (s.s.) mitochondrial genome, indicating females of the species at its limit of distribution (Fig. 7b).

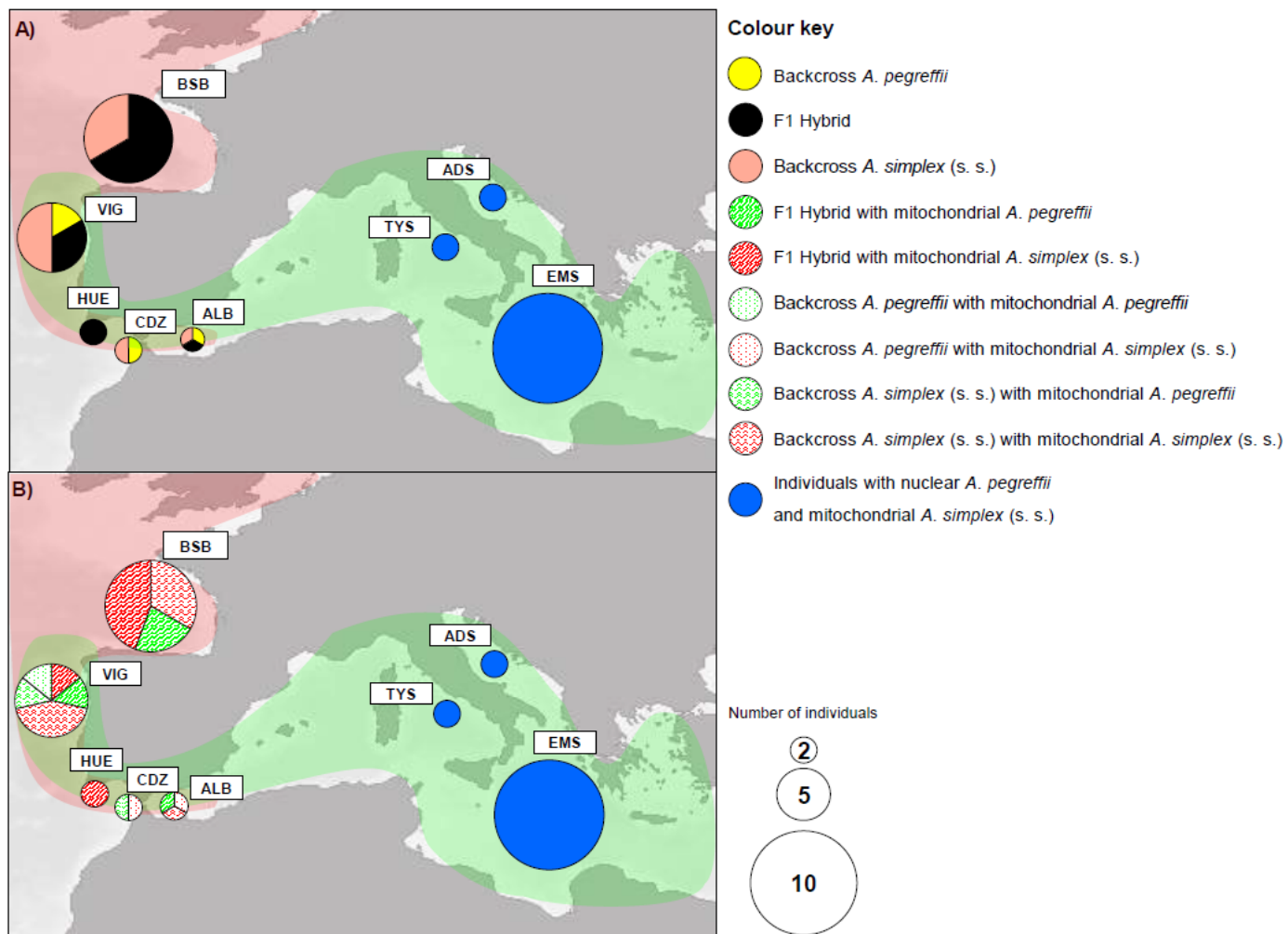


Figure 7. Spatial distribution and relative proportions of the F1 hybrids, backcrosses and mitochondrial introgressed specimens detected, in the present study, by multimarker approach according their nuclear genotype (A) as inferred from STRUCTURE and NEWHYBRIDS (see Figure 5), also showing their maternal lineage (B)..

In addition, the combined effect of different proportions of mature worms of the two species coinfecting the same definitive host in a contact area, coupled with their differential timing of sexual maturity, could be at the base of occasional hybridization events. Indeed, a possible differential reproductive timing could influence the fertilization success in the two closely related species, by preventing interspecific mating as a *pre-copula* mechanism of reproductive isolation. For instance, *in vitro* culture experimental works, carried out from L3 to reproductive adult stage of the two species, have demonstrated that females of *A. pegreffii* reach maturity and begin egg production from the 25th day of culture (Moratal *et al.* 2023), while it has been observed that males of *A. pegreffii* reach maturity between the 40th and 50th day (Nascetti *et al.* 1986; Mattiucci *et al.* unpublished data). In contrast, both males and females of *A. simplex* (s.s.) reach reproductive stage around the 30th day of *in vitro* culture (Nascetti *et al.* 1986; Mattiucci *et al.* unpublished data).

Thus, the combined effect of different proportions of mature worms of the two species coinfecting the same definitive host in a contact area, coupled with their differential timing of sexual maturity, could be at the base of occasional hybridization events.

The findings reported in the present study seem suggest that some mechanisms of reproductive isolation, both *pre-* and *post-copula*, might be not fully efficient in overlapping areas. Generally speaking, hybridization events may give rise to F1 hybrids with low viability and sterility, thereby preventing gene flow between the two parental taxa. It is also possible that F1 hybrids are seldomly able to backcross with one of the two parental species, giving rise to F_n generation of hybrids. This can result in either 50% genome admixture in F1 hybridization or to introgression (i.e. the introduction of single genes from one species into another closely related species through repeated backcrossing events). In our sample, the *Fst* values observed between the parental taxa and backcrosses at nuclear level suggest that, despite on-going hybridization in the contact zone between the two closely related species, some *post-copula* mechanisms of reproductive isolation may also be present, maintaining the evolutionary boundary of the species.

However, hybrid zones involve amalgamations of independently evolving genomes, and various sexual asymmetries can be disclosed by joint data from cytoplasmatic and nuclear markers. For instance, the occurrence of mito-nuclear discordance (Table 4), demonstrated by 16 specimens from the eastern Mediterranean Sea showing the nuclear genome of the parental *A. pegreffii* with the cytoplasmic genome of the parental *A. simplex* (s.s.). This mito-nuclear discordance indicates mitochondrial introgression in the genome of *A. pegreffii* in this area. In several hybrid zones, the pronounced discordance across nuclear and mitochondrial loci appears to reflect different historical appearance of introgression (Porretta and Canestrelli 2022). Indeed, a common phenomenon is cytoplasmic "capture", where mtDNA genotypes characteristic of one species occurs against the predominant nuclear background of the interacting species. The occurrence of

interspecies introgression of mitochondrial genomes was detected in other parasitic nematodes, such as between *Trichinella spiralis* and *T. britovi* (Franssen *et al.*, 2015). This phenomenon, here observed in L3 stage larvae of *A. pegreffii* from eastern Mediterranean samples at a conspicuous rate, was not previously described in other allopatric and sympatric areas of the two *Anisakis* species. However, similar findings of mito-nuclear discordance in *Anisakis* individuals - despite based on only one nuclear gene locus (i.e ITS regions of rDNA) – were reported by Mladineo *et al.* (2017b) from the Adriatic Sea (Croatian coast). It is important to note that in our sample only few specimens (N = 4) displayed the most common haplotype (i.e H7), which is shared with other populations of the parental taxon *A. simplex* (s.s.). In contrast, the remaining haplotypes (N = 12) were private, occurring only in those introgressed specimens. Among these, H18 and H19 resulted to be private haplotypes, never found in other sympatric or allopatric populations of the two parental taxa (Figs. 6 and 7b), while the H20, frequently found in our samples, was previously detected from the Tunisian coast of Mediterranean Sea, and from Adriatic Sea. This pattern suggests that this finding would be the result of both past and/or paleo-introgression hybridization phenomena that occurred in the genome of the interacting species. It is hypothesized that limited hybridization between the two taxa had occurred over several episodes. Past and/or paleo-introgression phenomena could be attributed to repeated immigration of the parental taxon *A. simplex* (s.s.) from the Atlantic Ocean, which might contribute to the observed mito-nuclear discordance. Supporting this hypothesis is the finding of private haplotypes found in the Mediterranean Sea, which form a distinct mitochondrial lineage from other haplotypes (Fig. 6). These could represent remnant haplotype lineages which survived in different micro-refugia in Mediterranean waters, following a secondary contact between the two separated lineages occurred during the Plio-Pleistocene period (5.3–2.0 Ma). Similar paleo-introgression inferences have been extensively reported for other marine invertebrate taxa in the Mediterranean Sea (Schmitt *et al.* 2021).

Another possible explanation for this finding is that mtDNA tends to introgress more readily than nuclear DNA, being genes contributing to reproductive isolation mechanisms generally located in the latter, rather than in mitochondrial DNA (Barton and Jones, 1993). Among additional factors driving the nuclear and cytoplasmic introgression, the asymmetry between fitness of males and females, can lead to differential exchange for nuclear and cytoplasmic loci. Sex-biased processes or dissortative preference in female mate choice could play a role in mitochondrial introgression. For instance, sperm-egg recognition proteins could be generally highly specialized, making interspecific zygote formation nearly precluded between the two species. In gonochoristic parasites, it has been observed that sperms penetrating egg's jelly coat have proteins binding specifically with receptor present on the egg of its own species (Mehlhorn, 1988). This mechanism

has not been yet described in anisakid parasites, where the direct contact between females and males in the definitive host and copulation, likely ensures sperm transfer. However, the asymmetry of hybrid fertility has been evoked in some animals (Mastrantonio *et al.*, 2019), to explain instances where mtDNA appears to cross species boundaries. This may occur if fertile females leave open the possibility for interspecific cytoplasmic recombination. Hybridization between heterospecific individuals has been documented in invertebrates as playing a role even in promoting the paternal leakage and mitochondrial heteroplasmy (Mastrantonio *et al.* 2019), where mitochondrial genomes from both parental species can occur within an individual due to relax mechanisms in the egg-sperm recognition. In this context, the possible occurrence of heteroplasmy between the two *Anisakis* species, behind the mtDNA introgression, can be also considered. In theory, significant genomic divergence can be accumulated during the process of speciation, creating strong barriers to gene flow and leaving other genomic regions, such as the mitochondrial genome, more permeable to introgression (Michel *et al.* 2010). To clarify this phenomenon, a complete cytoplasmic genome analysis and a linkage map would be also necessary to determine the impact of any genomic rearrangements of the two species in their contact areas. .

However, the maintenance of *A. pegreffii* and *A. simplex* (s.s.) as distinct phylogenetic lineages, coupled with the high level of differentiation between backcrosses and parental taxa observed in this study, despite recurrent hybridization in the contact area, suggests that certain microevolutionary processes likely act as barriers to gene flow (e.g., selection against hybrids, assortative mating, genomic incompatibility between species).

F1 hybrids may exhibit reduced viability or fertility, with these effects potentially being more pronounced in one sex. Haldane (1922), hypothesized that in hybrid populations, if one sex is absent, rare, or sterile, it is typically the heterogametic sex. Consequently, ongoing introgression of nuclear genes can occur *via* either male or female hybrids. In our sample from the sympatric areas, 1 adult female worm was found to have a F1 genome, possessing *A. pegreffii* mtDNA (Fig.7b), whereas the most identified F1 hybrids and backcross individuals were L3 stage larvae, possessing a *A. simplex* (s.s.) (N= 16) or *A. pegreffii* (N= 6) mtDNA (Fig. 7b). According to the SSRs sex-linked loci, those 16 third stage L3 larvae, i.e. N= 10 F1, *plus* N= 3 backcrosses, were all females, while N= 9 backcrosses were males. According to the same sex-linked loci, the sex assignment to the mitochondrial introgressed individuals resulted to be N= 3 females and N= 3 males. Thus, in our case, since most of the F1 hybrids were female worms, ongoing hybridization seems to be predominantly maintained by females. This finding seems to align with Haldane's rule. Various causes of Haldane's rule, such as post-zygotic sterility and inviability of hybrids, have been proposed for several vertebrates and invertebrates, including chromosomal rearrangements, incompatibility between sex chromosomes and autosomes, and the dominance theory of sex-linked

genes (Cowell, 2022). These processes may contribute to maintaining reproductive boundaries between the two parasite species here studied.

Future studies on these nematode parasites, with Mendelian inheritance, will enhance our understanding how mechanisms of reproductive isolation between closely related species drive the evolutionary force which has led to the high success of their offsprings, even in sympatric and syntopic conditions. This would also explain the mechanisms that maintain species reproductive barriers, as well the relative low frequency of hybrids (here found to be around the 5%) observed in this overlapping populations of the two *Anisakis* species. Further investigation of selective pressures and patterns of genomic introgression will be necessary to determine the phenotypic traits involved in reproductive isolation between the two species, and the nature of hybridization between them.

Hybridization can also represent a fertile field for natural selection, promoting certain genotypes/haplotypes producing more potential adaptive phenotypes with respect to the parental species in determined conditions. A "transgressive hybrid" refers to hybrids expressing phenotypes which are not resident in either the two parental taxa. This can result to the fixation of more adaptive alleles in certain genes, potentially related to new environmental variables. Indeed, since hybridization can generate both phenotypic and genetic variants, the occurrence of natural hybrids between interacting species of parasites may be linked to the presence of emerging and rapidly changing environmental factors that favour the survival of hybrid phenotypes. It could also happen that, though hybrids may be poorly adapted to either parental species habitats, they can sometimes outcompete parental species in changing habitats, conferring a selective advantage (King *et al.* 2015). In this respect, introgressive hybridization could play a key role also in the microevolutionary ecology of the *Anisakis* species in the contact zone here studied.

The current distribution of the two parental *Anisakis* species in European waters (Mattiucci *et al.* 2018; Diez *et al.* 2024) is shaped by complex biotic and abiotic factors. Water temperature and salinity play a crucial role in determining egg hatching and the survival of the first larval stages (Gomes *et al.*, 2022). The parental genomes of the two *Anisakis* species would provide a set of genes having a selective advantage in relation to these variables, defining their temperature and salinity tolerance limits. In this context, the mixed genome of introgressed individuals may be favored over the parental genomes at the boundaries of their distribution ranges, offering a broader temperature tolerance to their free-living stage larvae. Interestingly, this study found that the differential pattern of hybridization along the northern Iberian Atlantic coast is mainly composed of F1 hybrids and backcrossed individuals with the parental *A. simplex* (s.s.) this area represents the northern limit of the geographical distribution of the pure parental taxon, *A. pegreffii* (Mattiucci *et al.* 2018; Diez *et al.* 2022) (Fig. 7a). The opposite trend was observed along the southern part

of Atlantic Iberian coast and Alboran Sea waters, where, aside from F1 hybrids, backcrosses with the parental *A. pegreffii* were identified, mostly showing a *A. simplex* (s.s.) mitochondrial inheritance. This region represents the southern limit of the geographic distribution of pure parental taxon *A. simplex* (s.s.) (Fig. 7a). According to this finding, we could hypothesize that the new gene combinations found in F1 and Fn individuals may enable them to tolerate marine environments that would be unfavourable to their parental species.

As the distribution of the two species of *Anisakis* in European waters may shift under climate change (Levsen *et al.* 2018; Diez *et al.* 2022; Palomba *et al.* 2023), eventually enlarging their contact area, the likelihood of hybridization events could increase. This could lead either to a lower fitness of the introgressed individuals following the admixture, or to the enhancement of the evolutionary adaptation of the hybrid genotypes to new local conditions (King *et al.* 2015).

Finally, concerning the zoonotic role of these parasites, no data are so far available about the findings of hybrid genotypes identified as agents of human anisakiasis. So far, only two markers (i.e. ITS region of rDNA and/or mtDNA *cox2*) have been used to identify larval stages of etiological agents of these cases (Mattiucci *et al.* 2018; 2023; Shamsi *et al.* 2023). Future studies, based on genotyping of larval stages causing human anisakiasis using a broader set of nuclear markers, will provide a more comprehensive genetic characterization of the larval individuals responsible for the zoonotic disease.

3.5 Conclusions

The present work demonstrates that contemporary hybridization between the parasite taxa *A. pegreffii* and *A. simplex* (s.s.) occurs in the contact marine areas here studied, and that viable F1 hybrids have contributed to genomic introgression within the parental lineages. Since most of the hybrid individuals were identified as females based on sex-linked SSR loci, it can be hypothesized that ongoing hybridization is predominantly maintained by females in the sympatric area studied. The present study also shows the existence of *A. simplex* (s.s.) mitochondrial introgression in *A. pegreffii* from the Mediterranean Sea waters, not previously detected in other allopatric populations of the parasite species, supporting the hypothesis of paleointrogression events occurred in these waters.

The useful application of a multilocus nuclear genotyping approach and a Bayesian analysis to investigate the genetic structure of the sister taxa included in complex of species in sympatric areas, as well as to assign to those specific hybrid categories each individual specimen has been provided. Further, it has shown that the use of the only mitochondrial gene locus (i.e. mtDNA *cox2*) in the genetic/molecular identification of these parasites, especially when dealing with populations of these parasite species collected from their contact areas, can lead to the misidentification of specimens, due to the occurrence of nuclear hybridization and mitochondrial introgression phenomena.

As the distribution of the two species of *Anisakis* in European waters can shift under climate change eventually enlarging their contact area, the likelihood of hybridization events could increase. This could lead either to a lower fitness of the introgressed individuals following the admixture, or to the enhancement of the evolutionary adaptation of the hybrid genotypes to new local conditions. Thus, monitoring the hybridization and introgression phenomena between the two *Anisakis* species in this sympatric area, would represent a tool for monitoring changes in the geographical distribution of the two species in NE Atlantic waters, under a sea temperature increase scenario.

Finally, further investigation may reveal that hybrid individuals between *A. pegreffii* and *A. simplex* (s.s.) could both alter the host-parasite co-evolutionary adaptation with their natural hosts, and exhibit differential pathogenesis pathways, including those affecting accidental hosts (e.g., human infection).

4. CONCLUDING REMARKS

In this doctoral thesis, we investigated the possible role of anisakid nematodes as ecological indicators of marine ecosystems stability, employing a genetic approach to assess species diversity, genetic structure, and population dynamics across temporal and spatial scales, as well as admixture patterns, under varying ecological conditions. Paper no. 1, we explored the genetic variability of *Contracaecum osculatum* sp. B in the Arctic and sub-Arctic regions, assessing its potential role as a model species for monitoring trophic web stability over a temporal scale. By analysing both historical (1985–86) and contemporary (2021–22) samples, we utilized novel microsatellite markers along with mitochondrial DNA sequencing to detect changes in genetic diversity over approximately four decades. Our findings revealed shifts in genetic variability that correlated with fluctuations in host populations and broader environmental changes, likely influenced by human-related ecosystem disturbances. The results underscored the importance of long-term genetic monitoring to understand the impacts of global change on marine trophic interactions, demonstrating that parasites may serve as valuable sentinels for assessing ecological stability through time.

Paper no. 2, we investigated the hybridization and introgression phenomena occurring between *Anisakis pegreffii* and *Anisakis simplex* (s.s.) in their sympatric zone along the Atlantic Iberian coast and Mediterranean waters. By employing a multilocus genotyping approach, integrating newly developed and fixed nuclear and mitochondrial markers, we demonstrated the occurrence of contemporary hybridization, with viable F1 hybrids contributing to introgression phenomena. Additionally, we identified mitochondrial introgression of *A. simplex* (s.s.) within *A. pegreffii* populations in the Mediterranean, suggesting past hybridization events that may have shaped the genetic structure of these populations. This study provides the evidence that hybridization is an ongoing process with potential broader implications. Indeed, our findings suggest that monitoring the occurrence of these phenomena in sympatric zones will be essential in understanding how these species respond to shifting climate conditions, likely expand their contact areas.

Together, these studies highlight the ecological significance of anisakid nematodes in marine ecosystems and demonstrate the power of genetic tools in monitoring biodiversity and evolutionary processes. The use of molecular markers is crucial for conservation efforts, enabling us to predict how environmental changes may affect the stability of marine trophic webs through monitoring how marine parasites respond to anthropogenic and climatic pressures.

Future studies should expand on interdisciplinary approaches to further elucidate the spatial and temporal dynamics dealt here. Increasing genomic resolution through whole-genome sequencing and/or integrating genetic data with oceanographic and environmental variables will enhance our ability to understand the impacts of global change on marine biodiversity.

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