

**Integrating parasites data and host genetic structure in the frame of a holistic approach for  
stock assessment in Mediterranean Sea fish species**

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**RUNNING HEAD:** *Parasites in fish host subpopulations*

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## SUMMARY

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The unique marine environmental conditions of the Mediterranean Sea together with its historical characteristics and its connection with the Atlantic Ocean make the stock assessment of pelagic and demersal fish species of this basin; a challenging topic, which has been investigated by different approaches. Most information on stock identification of Mediterranean fisheries has been collected in the last years by applying biological and morphometric traits, artificial tags, otoliths shape, and genetic methodologies. However, less effort has been given to the use of parasites as biomarkers. This review focuses on the researches carried out, in recent years, on the simultaneous use of population genetic analysis and parasites as biological tags to assess population structuring of some pelagic and demersal fish species from the Mediterranean Sea, also in comparison with populations of the same fish species from the Atlantic Ocean. Congruence and incongruence in depicting fish stocks structure according to the two different data sets,( i.e. fish genetics and parasites), are presented and discussed in the light of the biological features of the species. A significative association between the population genetics (allozyme markers) and biological (*Anisakis* spp. parasites) data sets acquired in some fish samples from the Mediterranean Sea and Atlantic Ocean was obtained through a meta-analysis approach performed by generalised Procruster Rotation (PR). Using this approach was highlighted the stock structure of the demersal and pelagic fish case studies, represented by the European hake, *Merluccius merluccius*, the horse-mackerel *Trachurus trachurus* and the swordfish *Xiphias gladius*. The results obtained suggested that the combined data sets produced stronger information on the fish population structure, rather than that indicated by a single data set. Some discordance between the two data sets was shown in the case of *T. trachurus*, where allozymes accounted for by a less structuring power with respect to the parasites, these latter

accounting for by most of the differences found at the meta-population level. Incongruences could be due to the features of the two kinds of data. Indeed, while the genetic studies on fish host populations can detect changes over an evolutionary time scale, the occurrence and composition of the parasite fauna in fish populations, as other fish biological parameters, are very good and suitable biomarkers, when considering a fish stock in a temporal (as related to the fish life-history) and spatial (as related to the geographic region) scale.

Future studies on the host-parasites co-phylogeographical analysis, including population genetic structure of suitable parasites as biomarkers and that of their fish host inferred from the same polymorphic genes, could represent a further tool in a holistic approach, able to infer stocks structure of commercially important fish species over their range of distribution.

**KEYWORDS:** Mediterranean Sea, biological tags, *Anisakis*, fish genetic structure, genetic/molecular markers, stock assessment

## INTRODUCTION

A stock can be defined as a group of individuals of a particular fish species whose genetic and life-history characteristics are more similar each other, rather than to the other subpopulations (or stocks) (Waldmann, 2005). The stock concept has been introduced to better understand the state of over-exploited fish subpopulations, and to highlight which areas within the species range result at higher risk of being over-fished. The correct identification of biological stocks is an useful approach in the management of economically important fish species, since it is the unit of both management practices and conservation measures. Indeed, when a stock of a particular fish species has been delineated, including the determination of its boundary between conspecific subpopulations that is characterised by its abundance, growth, reproduction, spawning, and genetic diversity, then the application of conservation measures may be applied (Waples, 1998).

Marine fish have been thought to have little genetic subdivision among geographically isolated populations (Ward *et al.*, 1994). Indeed the gene flow, throughout the passive transport of pelagic eggs and larvae, or by active adult migration over large distances, contributes to maintain the genetic homogeneity of large and stable fish populations of the same species over large geographical scale. Recent data have partially modified this feeling, highlighting geographic structure in many different fish species (Coven and Sponaugle, 2009). Although the use of genetic/molecular data has become the common basic method to identify fish population structure, allowing evaluating the genetic differentiation and gene flow between subpopulations across the geographical distribution of a fish species, the assessment of fish stock is based nowadays on a holistic approach (Waldmann, 1999). This includes the use of fish morphometrics, artificial tags, fish genetics and other biological tags to identify stocks structure. Among the biological tags, the parasite-based methodology (including parasite community structure of the fish species along its geographical distribution, parasitic infection levels, genetic/molecular

characterization of parasite species) represents an important aspect in defining a fish stock.

Indeed, parasites can provide ecological information on origin, migration, nursery ground and life-history of the fish species (Thomas *et al.*, 1996).

The basic principle of the use of parasites as biological tag is that a fish became infected by that parasites species only when it is in the endemic area of that parasite. An endemic area of the parasite is the geographical region where the abiotic (temperature, salinity) and biotic (presence of suitable intermediate and definitive hosts) factors are suitable for the transmission and completion of its life-cycle. Thus, we could assume that when a fish population is found infected by a parasite species, it means that the fish has spent part of its life-history in the endemic area of the parasite, where fish behaviour and feeding habits could have lead to a different infection levels by that parasite species. As a consequence, when the parasite fauna of two populations of the same fish species sampled from two different geographical areas is different, it means that the life-history of those fish samples were different.

Basically, a parasite can be used as suitable biological tag of fish stock when its geographical distribution and life-cycle are known, and when the parasite's residence time in the host is long enough as the life-history of the fish hosts. In this sense, the parasite as biological marker reflects the geographic origin of the fish population on a spatial scale. This is the main reason why concordant and discordant results in the existence of different stocks of a fish species between the genetic/molecular data sets on the fish and the results about the use of some parasites as biological tags, may occur. Indeed, the genetic/molecular markers define the stock on the evolutionary temporal scale, while the parasite taxa characterize the stock on a spatial/geographical scale level.

This paper is dealing with a revision of the researches carried out on the use of parasites as biological markers to assess stocks structure of some pelagic and demersal fish species from the Mediterranean Sea. Specifically, it focuses on the researches carried out, in recent years, on the simultaneous use of population genetics analysis and biological markers (parasites) to assess sub-

populations structuring of some pelagic and demersal fish species from the Mediterranean Sea, in comparison with populations of the same fish species from the Atlantic Ocean.

## THE MEDITERRANEAN SEA AND FISHERIES

The Mediterranean Sea is a semi-closed sea with a surface of about 3 million Km<sup>2</sup> (including the Black sea) representing the 0.8% of the total world marine surface. Its range of latitude is from 30°N to 46°N, in a temperate zone of the Northern Hemisphere. It consists of two connected mid-latitude basins, the Western (WM) and the Eastern Mediterranean (EM) separated by the shallow (400m) sill of the Sicily Channel, and is characterised by a limited water exchange with the Atlantic Ocean. The thermoaline circulation of the Mediterranean Sea is generally described as an open basin-wide cell, resulting in the transformation of surface water of Atlantic origin, on its eastward propagation into Levantine Intermediate Water (LIW), and two closed secondary cells, one in the Western Mediterranean (WM) and the other in the Eastern Mediterranean (EM), which involve the transformation of the surface and intermediate water into Eastern and Western deep waters (Gačić *et al.*, 2012). Water mass is stratified during the summer, however the temperature is around 13°C in the deep water (below the 400 m) all over the year. The absence of precipitation during the summer period is the main characteristic of the Mediterranean Sea. The loss of water through the evaporation is partially balanced by the contribution of Atlantic water throughout the Gibraltar Strait. In the Mediterranean Sea the evaporation exceeds precipitation and run-off: the salinity increase, due to evaporation over the Mediterranean surface, is compensated by an outflow of salty water into the Atlantic Ocean. Cooling in winter causes convection to intermediate depths forming the Levantine Intermediate Water (LIW). This salty intermediate water returns to the west underneath the AW and becomes the main component of the Mediterranean Outflow to the Atlantic. The LIW is a crucial component of the Mediterranean thermohaline, because it is the warmest and saltiest Mediterranean water formed in large amount,

and because it mainly flows westward along the northern continental slopes of both basins, influencing the North Atlantic Deep water formation (Gačić *et al.*, 2012).

The Mediterranean and Atlantic regions have been long-recognised marine zoogeographic provinces. The 45% of the Mediterranean fish species that occurs also in the Atlantic are retained to have invaded the Mediterranean since the opening of the Gibraltar Strait, in the early Pliocene (Quignard, 1978). The dramatic paleogeographical and paleoclimatic events have played a crucial role in the evolutionary history of many marine species in the Mediterranean region (Valsecchi, 2005). The closure between the Atlantic Ocean and the Mediterranean Sea, which occurred between about 5.96 and 5.33 mya, determined the Messinian salinity crisis. The re-opening of the Gibraltar Strait (about 5.33 mya) was characterised by the colonization events.

Actually, the Mediterranean fisheries represents a small proportion of the European production, only the 20% of the total catch; the mean annual landings (mainly sold as fresh) have been around 1.5 million of tons in the last decade. The Mediterranean counts 21 border countries, (excluding the Black Sea); among them, the European countries (Spain, France, Greece and Italy) represent the one third of the Mediterranean Sea coastline. From a socio-economic point of view, fishing activity is highly diverse and varies by geographical areas and countries. This is mainly related to different environmental conditions and is due to various socio-economic conditions in the countries whose fisheries insist on the Mediterranean Sea. The Mediterranean fisheries is characterised by fragmented fleets, usually composed by relatively small vessels, multi-species catches, and a large number of landing sites. In general, the lack of connection between fish stocks assessment and management is the main problem for the Mediterranean fisheries. It occurs because there are scarce available historical data on landings and, given the complexity and diversity of the Mediterranean fisheries, insufficient data are available for the assessment of most fish species (Leonart and Maynou, 2003). The 95% of the fish stocks are overfished, which means that catches are higher than they should be in order to make the fish stocks grow to

sustainable stock sizes. The management of the Mediterranean fisheries is based mainly on the effort control. No TACs are implemented (except for the bluefin tuna and swordfish ), nor other types of adaptive management Other technical measures, such as the minimum landing sizes and minimum mesh sizes, are also implemented, but they are lower than in the Atlantic fisheries (Lleonart and Maynou, 2003).

Large pelagic, small pelagic and demersal fisheries are the three main different groups of fish resources in the Mediterranean Sea. The bluefin tuna (*Thunnus thynnus*) and the swordfish (*Xiphias gladius*) are the most important large pelagic species in the Mediterranean Sea; they represent around the 4% of the total reported landings. ICCAT (International Commission of Atlantic Tunas) recognises the existence of a single stock of bluefin tuna for the North-Eastern Atlantic Ocean. Swordfish is the second large pelagic fish species; ICCAT recognises the existence so far of a single stock unit of swordfish in the Mediterranean Sea, distinct from those of the Atlantic Ocean (see paragraph devoted to *X. gladius*). Small (anchovies, sardine) and medium-size (mackerels, horse mackerel, bogue, ect.) pelagic fish are the main contributors (more than 50%) to the total landings in the Mediterranean. Demersal species in the Mediterranean Sea include more than 100 species; they have commercial value, despite the large number of demersal fish species, according to the FAO, and they represent only the 3% of the total catches (Lleonart and Maynou, 2003).

## CURRENT METHODOLOGIES USED FOR THE IDENTIFICATION OF MEDITERRANEAN FISH STOCK UNITS

Definition of fish unit stocks has been largely absent and in some cases still problematic in the Mediterranean fisheries (Caddy, 1995). The Scientific Advisory Committee of the GFCM (General Fisheries Commission for the Mediterranean) has established management areas based on political and statistical consideration, rather than on biological ones (FAO, 1999). The



Mediterranean basin has been split in 27 Geographical Subareas (GSA) for statistical purposes and data on captures and production are reported accordingly (GFCM, 2012). The availability of appropriate data is considered one of the main shortcomings in the assessment of the Mediterranean fisheries. There is a large amount of reliable data but they are not often adequate to fit standard stock assessment methods. However, scientists have tried to assess the fisheries with several tools, using available data. Analytical methods based on population dynamics (long time series of size/age relationships, growth, etc.) have been applied to different stocks of demersal, small pelagic and large pelagic. However, the single approach method has been not considered as the main suitable in fish stock assessment.

On the other hand, the marine environmental condition and the historical data of the Mediterranean Sea basin make the fish stock assessment of pelagic and demersal fish species, with a wide geographical distribution, as a challenging argument to be investigated by different approaches. Generally, marine fish stock identification has been challenging, due to the low physical barriers and ocean current existing in the marine realm, that facilitate extensive gene flow among fish populations. In absence of physical barriers or great geographical distances, marine fish species could exhibit cryptic population structuring based on differences in their recruitment, spawning behaviour, mechanisms of retention of larval stages and general life-history strategies and traits (Knutsen, 2003). However, because the Mediterranean and Atlantic populations of marine organisms has been isolated due to the sea level changes during the glacial maxima and then reconnected several times in the past, the genetic divergence of populations during isolation followed by secondary contacts after the re-opening of the Gibraltar Strait, accompanied by a reduced gene flow between the geographically divergent populations, could have led to the present occurrence of populations substructuring observed in some fish species across the Mediterranean and Atlantic waters. Further, the Almeria-Oran oceanographic front and the occurrence of a defined hydrographic barrier have been suggested to be responsible for its

position as the boundary between Mediterranean and Atlantic populations of several marine organisms (Quesada *et al.*, 1995; Pannacciulli *et al.*, 1997). Thus, Gibraltar Strait is not regarded as the main barrier between the Mediterranean and the Atlantic marine populations. The transport of North Atlantic water through a discontinuous one-way surface current flow throughout the Gibraltar Strait into the Alboran Sea is also documented (Tintore *et al.*, 1988; Patarnello *et al.*, 2007).

Three main hypotheses may explain the subpopulation structuring in marine pelagic and demersal fish of the Mediterranean fish resources: *i*) environmental factors, including the past sea level changes, past barriers and present oceanic barriers, such as oceanic currents responsible for the mixing or interrupting gene flow among populations; *ii*) increasing of geographical distances; *iii*) life-history traits, including potential for larval dispersal, differential homing and spawning areas behaviour, larval retention in coastal zones.

Generally speaking, transition zones are expected to play an important role in limiting the gene flow among populations, thus promoting their genetic substructuring. The existence of the Atlantic Ocean-Mediterranean Sea transition has been shown for several fish species such as the swordfish, *Xiphias gladius* (Kotoulas *et al.*, 1995; Kasapidis, *et al.*, 2007; Cimmaruta *et al.*, 1998; Chow *et al.*, 1997; 2007; Alvarado-Bremer *et al.*, 2005a; 2005b), *Dentex dentex* (Bargelloni *et al.*, 2003), mullets *Mugil cephalus* (Crosetti *et al.*, 1994), sand goby, *Pomatoschistus minutus* (Stefanni and Thorley, 2003), Mediterranean sea bass, *Dicentrarchus labrax* (Naciri *et al.*, 1999; Bahri-Sfar *et al.*, 2000) and poor cod, *Trisopterus minutus capelanus* (Mattiangeli *et al.*, 2003). Moreover, an eastern-western fragmentation around the Siculo-Tunisian strait has been suggested in *Dicentrarchus labrax* (Bahri-Sfar *et al.*, 2000) and in *Xiphias gladius* (Kasapidis, *et al.*, 2007). Similarly, differences in specific hydrological, topographical and oceanographic features (depth, temperature, salinity) between the northern and southern part of the Adriatic Sea seem to be responsible for the genetic isolation of Adriatic sardine populations from the rest of the

Mediterranean Sea (Alegria-Hernandez, 1986); these findings seem to be not supported by the application of some genetic markers (Tinti *et al.*, 2002), despite a subpopulation structuring has been suggested recently based on the microsatellites loci analysis between Adriatic and Ionian Sea sardines (Ruggeri *et al.*, 2013).

Most information on stock identification has been collected by applying morphological characteristics, morphometric traits, artificial tags, otoliths shape, and genetic methodologies. Indeed, in the last 15 years, genetic/molecular markers have become the most favoured method to assess fish population stock identity. Fishery scientists have applied many different types of genetic/molecular markers in the study of Mediterranean fish population structure, such as allozymes (Cimmaruta *et al.*, 1998; 2005; 2008), mitochondrial DNA loci (Tinti *et al.*, 2002), and, more recently, microsatellites loci (Ruggeri *et al.*, 2013, and reference therein) and single nucleotide polymorphisms (SNPs) (Milano *et al.*, 2014, and references therein). These are the most favourite markers to establish fish host stock structure, to assess their genetic differentiation and to estimate their genetic variability levels. Genetic studies complied to biological studies have been so far carried out for the stock definition and assessment in anchovy (Bembo *et al.*, 1995, 1996a; 1996b; Pla *et al.*, 1996; Tudela *et al.*, 1999), swordfish (Kotoulas *et al.*, 1995; Kasapidis, *et al.*, 2007; Cimmaruta *et al.*, 1998), hake (Roldan *et al.*, 1998; Cimmaruta *et al.*, 2005; Milano *et al.*, 2014); mackerel (Zardoya *et al.*, 2004), sardinas (Ruggeri *et al.*, 2013), mullets (Crosetti *et al.*, 1994 ).

The literature concerning the use of parasites as biomarkers in the fish stock assessment of Mediterranean fish resource is not very vast. Among the methodologies applied in this field, the morphological identification of the parasites species, the use of genetic/molecular markers for the parasites detection, combined with the estimation of parasitic infection levels and multivariate analyses of the parasite community composition, are those mainly used so far to that scope.

Power *et al.* (2005) used parasite infracommunity as predictors of harvest location of bogue, *Boops boops*, in two fisheries off the Atlantic coast of Spain, compared with one off Spanish Mediterranean coast. Two parametric methods of classification were compared to other non-parametric methods to assign fish specimens to their fishery ground. Five predictor parasite species, including *Aphanurus stossichii*, *Baccinger isralensis*, *Hemiurus communis*, *Microcotyle erythrini* and *Lecithocladium excisum*, present at high abundance in the whole host population, were considered as suitable biomarkers for the classification of bogues collected from different geographical areas of the Atlantic *versus* the western Mediterranean Sea (Spanish coast).

Similar studies have been carried out on parasite distribution of the striped red mullet, *Mullus surmuletus*, one of the most abundant fish and widespread distributed in the coastal zone of the Mediterranean Sea and along the sublitoral zone of the North East Atlantic Ocean. Prevalence and abundance of three long-lived parasite taxa differed significantly among three localities off the Spanish Mediterranean coast (Ferrer-Castello *et al.*, 2007), indicating their potential as stock indicators of the striped red mullet, *Mullus surmuletus*. A cluster analysis based on prevalence and MANOVA on abundance data sets indicated strong inter-sample variability, even within the same locality, with poor spatial segregation among samples. A linear discriminant analysis (LDA) based on the abundance of 17 parasite taxa correctly assigned over 80% of fish to their locality, indicating good and stable predictive power based on the parasitological data (Ferrer-Castello, *et al.*, 2007).

Klimpel *et al.* (2008) found regional differences in the distribution of the parasites taxa identified in fish host populations of *Mullus surmuletus* sampled from the Mediterranean Sea and along the sublitoral zone of the North East Atlantic Ocean, suggesting the possible role of the historical reasons of the Mediterranean Sea as an hypothesis to explain the differential distribution of the parasites species detected.

Regional differences in the infection levels by the larval nematodes of the genus *Anisakis* from anchovies, *Engraulis encrasicolus*, fished in different fishing grounds of the Mediterranean Sea have been suggested to be related to the existence of different populations of the fish species *Engraulis encrasicolus*, along its Mediterranean distribution (Rello *et al.*, 2009) The Authors found the prevalence of the infection by *Anisakis* spp. in anchovies from the Ligurian Sea to be significantly higher than in other three fishing areas from the southwestern Mediterranean Sea (Gulf of Lion, Catalonia coast, and Alboran Sea) (Rello *et al.*, 2009). In similar studies, no infection by *Anisakis* was found in anchovies fished from off the Tarragona (NE Spain) (Serracca *et al.*, 2013). Whereas, anchovy, *E. encrasicolus* caught in the Adriatic Sea off the Croatia coast showed moderately higher infection levels by *Anisakis* spp. larvae, with respect to other Mediterranean and Atlantic water which have shown strikingly lesser values of prevalence (Mladineo *et al.*, 2012). However, when considering the host population dynamics as having a possible role in the infection by the larval *Anisakis* spp., the fish host size has been not always taken into account to explain regional differences observed in parasitic infection values from the same fish species.

When carrying out a multimethodological approach to fish stocks assessment, which includes morphological and biological traits (including parasites) and the genetic structure of the fish host, it is fundamental to include fish samples from the whole range of the species and to use the same fish samples for all the methodologies applied (Abaunza *et al.* 2008a). Further, while the fish size is not a confounding variable to detect genetic population structure, the host size, especially in long-lived parasites – such as larval *Anisakis* are - could have a confounding effect. This explains why, whenever possible, the sample size of the fish host should be as much as possible homogenous and comparable for all the methodological approaches. However, it could also happen that when comparing fish population structure from different basin waters such as Mediterranean Sea and Atlantic Ocean, the fish size of the fish host, despite the same age, may be

different, due to other environmental variables (abiotic factors, food availability and overfishing). This is for instance the case of the Mediterranean and Atlantic populations of swordfish, *Xiphias gladius* (Tserpes and Tsimenides, 1995; Ehrhardt, 1992).

In addition, when choosing a parasite as the best suitable biomarker, in addition to the ecological characteristics of the parasite, it is also important to apply the most suitable method for its identification, especially when the parasite includes a complex of cryptic (or sibling) species. This is, for instance, the case of the larval nematodes of the *Anisakis simplex sensu lato* complex, which comprises so far three biological species, named as *A. pegreffii*, *A. simplex (s.s.)* and *A. berlandi* (see Mattiucci *et al.*, 1997, 2014). The third stage larvae of these three species, recovered from the fish host, show the morphotype indicated as *Anisakis* type I or *Anisakis* type II (*sensu* Berland, 1961), but they cannot be identified to their species level based on traditional morphological analysis. Among the genetic methodologies used for the species identification of *Anisakis* spp. larvae, the allozymes markers, as based on several diagnostic loci among the species of *Anisakis* so far genetically detected, are able to recognise the three species, at any of their life-history stages. In addition, because of the temporal and geographic stability of the allozyme data, they are used in assessing the distribution of the different recognised species of *Anisakis* in the same fish host captured from several geographic areas. Finally, in order to have a comprehensive pattern of distribution of a parasites species in a fish species along its geographical range, a large number of parasites (up to thousands) must be identified by molecular/genetic methodologies to their species level. This was, for instance, the case of larval *Anisakis* spp. identified in samples of *Merluccius merluccius* (Mattiucci, *et al.*, 2004), *Trachurus trachurus* (Mattiucci *et al.*, 2008) and *Xiphias gladius* (Mattiucci *et al.*, sub.). In addition, among the other molecular markers used for *Anisakis* detection (Mattiucci *et al.*, 2014), the high substitution rate of the mtDNA *cox2* sequences found so far in *Anisakis* spp. (Mattiucci *et al.*, 2014), also allows the use of

phylogeography of *Anisakis* spp. to provide support to the subpopulation structure, as inferred for its fish host.

In the next paragraphs particular attention has been given to review the use of some parasites species (i.e. larval nematode of the genus *Anisakis*) as biomarkers in three cases studies of opulations of large, small, pelagic and demersal fish species of the Mediterranean Sea within a multidisciplinary approach, including the use of genetic/molecular markers to detect fish host structure. The parasitological data sets concerning the occurrence of *Anisakis* spp. larvae are here reviewed in the context of the “fish stock assessment” as based on a “holistic approach”. Particular attention has been then given to the results achieved, in the recent years, on the populations genetic structure of three fish species (i.e. European hake, *Merluccius merluccius*, horse mackerel, *Trachurus trachurus* and swordfish *Xiphias gladius*) as inferred from allozyme markers, and on some parasite species (i.e. larval *Anisakis* spp.) which are the most suitable biological tags to characterize fish stocks.

Meta-analysis of parasites in their fish hosts was used to correlate the genetic data sets on the fish host and larval *Anisakis* as markers, as obtained on the same samples of the pelagic and demersal fish species collected in localities comprised in the range of distribution of the three species in Mediterranean and Atlantic waters.

Congruence and incongruence in depicting the stocks of those fish species according to the two different data sets, i.e. fish genetics and *Anisakis* parasites, are presented and discussed in the light of the biological features of the species.

## CASE STUDIES IN FISH STOCKS DEFINITION FROM THE MEDITERRANEAN SEA

### *Demersal fish - The case of the European hake, Merluccius merluccius*

The European hake is a demersal fish living in eastern Atlantic waters from the North Sea to the African coasts, including the Mediterranean and Black Sea. Hake fisheries are well developed

352 throughout the whole species range and the available data suggest that at least the Mediterranean  
353 population could be overexploited (GFCM, 2012).

354 Here we present data acquired on the pattern of distribution and abundance of different  
355 species of *Anisakis*, supporting the existence of different stocks of *Merluccius merluccius* sampled  
356 from several geographic areas covering its whole geographical range. The data were acquired in  
357 the framework of a multidisciplinary international project (GENHAKE), which was aimed at  
358 clarifying the stock structure of *M. merluccius* in European waters. The project included the  
359 analysis of biological and morphometric characteristics of the fish host, and the use of different  
360 nuclear genetic/molecular markers to define the stock structure of the fish species. The  
361 parasitological examination was carried out on the same individuals used for the genetic analyses  
362 and other biological studies.

363 Among the wide range of parasite taxa found in *M. merluccius* across its range of distribution,  
364 attention was focused on some selected parasites proved to be of particular value in stocks  
365 definition. They were the larval nematodes of the species *Anisakis*: they are long-lived parasites,  
366 occur at high frequency in the fish species throughout its geographical range and they are not  
367 pathogen to the fish host. Therefore, they respond to the main characteristics defining a parasite as  
368 a good “biomarker”. The relative proportions of larval specimens of different species of *Anisakis*  
369 in different sampling localities of *M. merluccius* allowed the recognition of Mediterranean and  
370 Atlantic stocks of European hake (Mattiucci *et al.*, 2004) (Fig.1a). *Anisakis pegreffii* was  
371 identified in all the hakes sampled from the Mediterranean Sea, except in the Levantine Sea  
372 (water off Cyprus coast). In the western Mediterranean Sea, *A. pegreffii* was the most prevalent  
373 and abundant species in Ligurian Sea; this finding seems to be related to the fact that this area is  
374 comprised in the Protected Marine Rea the Pelagos Sanctuary of Mediterranean Cetaceans, a  
375 Mediterranean protected Area inhabited by various dolphin species (Notarbartolo di Sciara *et al.*,  
376 2008), which are the main definitive hosts of *A. pegreffii* (Mattiucci and Nascetti, 2008;



Mattiucci *et al.*, 2014). This species occurred at high prevalence and density also in fish sampled from the eastern part of the Mediterranean Sea, such as in Ionian, Aegean and Cretan Sea. Interestingly, hakes from Levantine Sea waters harboured only the species *A. typica*, a species parasitic in many cetacean species in warm temperature and tropical waters (Mattiucci *et al.*, 2002). *A. physeteris* resulted as the most prevalent species in hakes from the western Mediterranean Sea off the Balearic Islands, Alicante and Malaga (Mattiucci *et al.*, 2004). The high proportion of this *Anisakis* species found in the fish from this deep area of the Mediterranean basin around Balearic Islands could be related to the presence of the main migration routes for the sperm whale *Physeter catodon*, the definitive host of *A. physeteris* (Mattiucci and Nascetti, 2008). However, to date hakes from the western Mediterranean Sea were not found infected with *A. simplex* (s.s.) (Mattiucci *et al.*, 2004) although this parasite species has been documented in some pelagic fish species, such as *Scomber scombrus* (Nascetti *et al.*, 1986) and *Trachurus trachurus* (Mattiucci *et al.*, 2008; see next paragraph) from some of the collecting areas. This finding supports the hypothesis that migrations of *M. merluccius* from and in Atlantic waters didn't occur. Conversely, in the north-east Atlantic hake samples from north of the Strait of Gibraltar, *A. simplex* (s.s.) was the most prevalent species, while it occurred off the Spanish Atlantic coast in sympatry with *A. pegreffii*. Indeed, hakes that exhibited mixed infections of *A. simplex* (s.s.) and *A. pegreffii* represented > 20% of the fish examined from the Atlantic coast of Galicia and 14% from the Bay of Biscay (Mattiucci *et al.*, 2004). Moving in the North-East Atlantic from the south of the Strait of Gibraltar, mixed infections of different species of *Anisakis* in hakes caught along the Atlantic coast of Morocco were observed. More than 22% of the fish from examined were found to be parasitized by five species of *Anisakis*: the major component species was *A. pegreffii*, followed by *A. physeteris*, *A. ziphidarum*, and, at lower percentage, *A. brevispiculata* and *A. paggiae* (Fig. 1a). Thus, according to the pattern of distribution of larval *Anisakis* spp. and their abundance in the hake samples, different populations of *M. merluccius* in

European waters (Mattiucci *et al.*, 2004) were designed by PCA analysis (Fig 1b). The larval distribution and abundance of the different species of *Anisakis* recognized in hakes from the different fishing grounds, indicate that: 1) two stocks of *M. merluccius* exist from Mediterranean and Atlantic waters, respectively exist; 2) in the North Atlantic area, at least two distinct subpopulations are present, one northern to the Gibraltar Strait (sample 3,6,7 Fig 1b) and another one (no.11, Fig b), from off the Atlantic coast of Morocco; 3) some substructuring in the western and eastern part of the Mediterranean hake populations seems to be recognised, with the population from Levantine Sea distinct from the other Mediterranean ones (Fig. 1b) (Mattiucci *et al.*, 2004, 2007). Interestingly, in the PCA analysis, the sample from Alboran Sea was more similar to Atlantic populations (Fig. 1b).

The parasitological findings are in agreement with the bulk of biological data, showing separate Mediterranean and the Atlantic stocks of European hake since they are known to live in markedly different environments and to differ in many biological and demographic features such as growth rate, spawning season and recruitment (Froese and Pauly, 2013).

In addition, the parasitological data were largely in accordance also with the genetic results obtained by using different molecular nuclear markers (allozymes, microsatellites, SNPs), showing a main subdivision between Atlantic and Mediterranean stocks (Roldan *et al.*, 1998; Lundy *et al.*, 1999; 2000; Cimmaruta *et al.*, 2005; Milano *et al.*, 2014). However, the molecular markers indicated that the boundary between the two stocks is located along the Almeria-Oran Front (AOF), instead of the Gibraltar Straits. Within the Atlantic basin, the hake genetic structure is coincident with both *Anisakis* findings and the ICES management areas: a northern and a southern stock of European hake are recognised in the Atlantic Ocean, each one corresponding to two ICES areas (International Council for the Exploration of the Sea).

Both allozymes and SNPs clearly identified a prominent role of selection in moulding the genetic stock structure between and within sea basins (Cimmaruta *et al.*, 2005; Milano *et al.*,

2014), indicating the sea water temperature and salinity as the main driving factors, shaping the genetic structure of a number of outlier loci. This finding suggests that the European hake represents a case study showing how marine environmental variables, such as temperature and salinity, are correlated to both the genetic structure of the fish host species, and to the pattern of distribution of the larval parasite *Anisakis* spp.

The high concordance among the different data sets in identifying the subpopulations of *M. merluccius* was gathered also from a generalized Procrustes rotation (PR) (Fig. 1c) which compares different ordinations of the European hake specimens as inferred from the combination of the genetic analysis (allozymes, data from Cimmaruta *et al.*, 2005) with the larval distribution of the different *Anisakis* species, as detected in the same subpopulations. The analysis showed a significant correlation (Procrustes  $r = 0.59$ ,  $P < 0.01$ ) between the distribution and abundance of the larval *Anisakis* and the pattern of fish-host genetic structure. The sampled specimens cluster in two well-defined groups corresponding to the Mediterranean (white circles) and Atlantic stocks (black circles) (Fig. 1c). Interestingly, at the PR analysis, the population from Alboran Sea (no.18, Fig. 1c) resulted more similar to the Atlantic ones, rather than to those from the Mediterranean basin. In addition, the eastern population from the Levantine Sea (no. 22, Fig.1c) resulted as distinct from the western Mediterranean populations (Fig. 1c). Both data sets are strongly contributing to the ordination recorded, with the molecular distance between samples accounting for about 93% of total ordination and the distance based on the parasitic burden of *Anisakis* for about 75%, and the final result is a resolution power higher than that of each single marker.

The results achieved in the case of European hake evidence how *Anisakis* spp. larvae are good biological markers in recognizing fish populations from different fishing grounds, given the assumption that these parasites are related to differences in life-history traits of their individual fish host. Indeed, the ecological differences of *Anisakis* spp., including their biogeography and life-cycles, reflect the environmental conditions and feeding behaviour of adult fish related to each single fishing ground in the Atlantic and Mediterranean Sea waters.

*Small pelagic fish - The case of the horse-mackerel, Trachurus trachurus*

The horse mackerel is a small carangid fish living on the continental shelf of North-Eastern Atlantic Ocean, from Norway to Cape Verde Islands, and in the Mediterranean Sea. Horse mackerel catches have a high relevance in fisheries, with landings from the Atlantic area strongly decreasing after 2000 and now partially recovering (Abaunza, *et al.*, 2008a; Fernandes and Cook, 2013; ICES, 2013). The horse mackerel is characterized by a high migratory ability and by a reproductive strategy with a rather extensive spawning season (four to eight months, according to the latitude) and an asynchronous maturation of oocytes, that mature and are spawned in a number of subsequent clutches (Abaunza *et al.*, 2003; Murta *et al.*, 2008). The association of high vagility and multiple spawning makes the boundaries of horse mackerel stocks very difficult to be defined, while its economic relevance makes this issue a crucial one.

In the recent years, the horse mackerel has been the target of a wide multimethodological research, aimed at identifying the biological stock of *T. trachurus* by simultaneously studying subpopulations of the fish species by means of different genetic/molecular markers, morphometry, life history traits and biological parameters, including parasites (Abaunza *et al.*, 2008a, 2008b). All the genetic/molecular data sets were concordant in describing a low level of differentiation among the Mediterranean and Atlantic populations, whilst a high genetic variation was found at the intra-population level. Both nuclear (allozymes and DNA microsatellites loci) and mitochondrial (ND4L and ND2 sequences analysis of mtDNA) markers found the larger part of the variation as distributed within the horse-mackerel populations sampled from the different fishing grounds rather than among them (Cimmaruta *et al.*, 2008; Comesaña *et al.*, 2008; Kasapidis and Magoulas, 2008).

The allozyme analysis evidenced an association between the levels of genetic differentiation and the geographic distance in the Atlantic Ocean (Isolation By Distance, IBD), making, however,

impossible to distinguish discrete stocks on the base of genetic data alone. These findings are in agreement with the biological features of the species, a vagile batch spawner, supporting the idea that the low genetic structuring of the fish species is related to a high gene flow due to the fish host behavior and mobility, rather than to the passive dispersion operated by marine gyres during the larval stage (Cimmaruta *et al.*, 2008).

Despite the low resolution power of genetic/molecular markers in the clearly distinguish the stocks of *T. trachurus* in European waters, several biological approaches (morphometrics, life history traits, otoliths and parasites) were able to detect discrete populations of the horse-mackerel (Abaunza *et al.*, 2008a). Indeed, the above mentioned techniques separated a Mediterranean stock from the Atlantic ones. A further subdivision in the NE Atlantic was observed: a “North Sea”, a “Western” and a “Southern” stock were identified. Also in the Mediterranean Sea, a western, a central and an eastern subpopulation were outlined (Fig.1a). In addition, the biological data acquired indicated an influence of the Atlantic populations on the western extreme (Alboran Sea) of the Mediterranean Sea (Abaunza *et al.*, 2008a, 2008c; MacKenzie *et al.*, 2008; Gordo *et al.*, 2008; Murta *et al.*, 2008; Stransky *et al.*, 2008; Mattiucci *et al.*, 2008).

The use of *Anisakis* parasites as biological tags gave concordant results with otoliths and morphometric data analysis (Mattiucci *et al.*, 2008), rather than with the genetic data sets. Of the five species of *Anisakis* identified in *T. trachurus*, *A. pegreffii* and *A. simplex* (*s.s.*) were found to be the dominant species in all the fish sampled along its geographical distribution, whereas other three species, i.e. *A. physeteris*, *A. typica* and *A. nascettii* (indicated as *Anisakis* sp. in Mattiucci *et al.*, 2008) were identified at very low percentages, respectively in the central Tyrrhenian Sea, the Cretan Sea and in the NE Atlantic fish samples (Fig. 2a). *A. pegreffii* and *A. simplex* (*s.s.*) showed statistically significant differences in their relative proportions in the horse mackerels samples throughout NE Atlantic and Mediterranean Sea. Indeed, *A. pegreffii* was identified as the main species parasitizing *T. trachurus* in the Mediterranean Sea; it was the only

species found in the Adriatic and Ionian Seas, while in the horse mackerels from Aegean Sea, *A. pegreffii* occurred in mixed infection with *A. typica* (1%). Similarly, in the western Mediterranean Sea, *A. pegreffii* was the dominant species, even if *A. physeteris* occurred in mixed infections in horse mackerel fished along the Tyrrhenian Sea coast (Fig. 2a). Mattiucci et al. (2008) found that the samples of *T. trachurus* fished in the Alboran Sea showed mixed infections by both the sibling species, *A. pegreffii* and *A. simplex* (s.s.) in almost equal proportion (Fig. 2a). Moving to the NE Atlantic Sea, the occurrence of *A. pegreffii* progressively decreased from the almost 87% from off the Portuguese coast of Algarve, to the 30% in the horse mackerels from off the Spanish Galician coast. *A. pegreffii* was rarely identified also from *T. trachurus* from off the south coast of Ireland and from the Bay of Biscay (Fig. 2a). The opposite trend was found in the occurrence of *A. simplex* (s.s.), which showed increasing proportions in the *T. trachurus* fished from off the Portuguese coast (almost 13%) to the Spanish Atlantic coast of Galicia (71%). It became the only species present in the fish species in the Norwegian Sea and along the western English Channel (Mattiucci et al., 2008) (Fig. 2a).

In *T. trachurus* fished along the Portuguese and Spanish Atlantic coast, a contact zone between *A. pegreffii* and *A. simplex* (s.s.), the two parasite species occurred at high percentage in mixed infection, at highest level, in 43% of the fish captured from off the North Portuguese coast. Similarly, in the Alboran Sea, the two species were found in syntopy in almost the 60% of the infected fish; while, most (35%) of the remaining fish examined from this basin water were found infected only by the species *A. pegreffii*. Further, according to Mattiucci et al., 2008, a few number of F1 hybrids between the two species *A. pegreffii* and *A. simplex* (s.s.), i.e. showing an heterozygote genotype at all the allozyme diagnostic loci, were recognized in the sympatric areas of the two parasites species (Mattiucci et al., 2008 and unpublished data). According to the genetically recognized species of *Anisakis* in the horse-mackerel (Mattiucci et al., 2008) and from abundance levels of infection, different populations of *T. trachurus* can be

distinguished, as shown in Fig 2a. Indeed, the statistical significant differences evidenced (Mattiucci *et al.*, 2008) in the relative proportions of *A. simplex* (s.s.) and *A. pegreffii* between the samples from the Mediterranean Sea (excluding Alboran Sea), and those from Atlantic, indicate a discrete structuring of subpopulations of *T. trachurus* in European basin waters (Fig. 2a). Moreover, the relative proportions and abundance levels of *A. pegreffii* in horse-mackerels fished in the Atlantic waters, seem to support the existence of a “southern” stock of *T. trachurus* along the Spanish, Portuguese coast, distinct from the “northern” stock, as represented by the areas of the Northern Sea, Norwegian Sea and South Ireland coast (Fig. 2a). Finally, it seems that the sample from the Alboran Sea (no.18, Fig. 2a and Fig. 2b), according to its larval *Anisakis* spp. composition, is clustering with the other Atlantic sample rather than with other Mediterranean Sea ones (Fig. 2b). This finding, and particularly the presence of a low percentage of fish infected only by *A. simplex* (s.s.) in the Alboran Sea, suggests that this population of horse-mackerel could be the result of a migration of this small pelagic fish species from Atlantic waters into this extreme western area of the Mediterranean sea, possibly mixing with the other Mediterranean Sea populations. The migrations of Atlantic horse mackerel into the western part of the Mediterranean Sea is also supported by the finding of the monogenean *Heteraxinoides atlanticus* in the same fish sample examined from the Alboran Sea (MaKenzie *et al.*, 2008), a monogenean species recognized in the Atlantic horse mackerel. Thus, in turn, the finding that the fish samples from the Alboran Sea are more similar, in their parasite fauna, to the Atlantic populations rather than to the Mediterranean ones, is supported by the existence of the oceanographic front, a particular water circulation, called as Almeria-Oran Front (AOF). This is an oceanographic front located from Almeria (Spain) to Oran (Morocco), consisting of inflowing Atlantic water which entering at the surface through the Gibraltar Strait, describes in the Alboran Sea a quasi-permanent anticyclonic gyre in the west and less variable on the eastern part. This oceanographic front exhibits a pronounced step temperature (1.4 C°) and salinity gradient (2ppt) over a distance of 2 km with an

average current speed of 40 cm/s flowing southeastward from the Spanish coast to the coast of North Africa (Tintore *et al.*, 1988). This water circulation could explain the finding of *A. simplex* (*s.s.*), a parasite species widespread between the 35°N and the Arctic Circle, in *T. trachurus* fished from the Alboran Sea. Those horse mackerels could have been infected by preying upon infected invertebrates and/or smaller pelagic fish species in the basin water of Almeria-Oran Front (AOF).

Thus, *T. trachurus* shows a low level of genetic differentiation between the Mediterranean and Atlantic populations, a stable genetic structure and a high level of genetic variability without apparent geographic structuring (Cimmaruta *et al.*, 2008; Kasapidis and Magoulas 2008; Comesana *et al.*, 2008). However, several other approaches (morphometrics and parasites) support the separation between the Mediterranean and Atlantic populations of *T. trachurus*, whilst parasites, morphometrics and life-history traits supported the existence of the “southern” and “northern” stocks in the Atlantic waters (Murta *et al.*, 2008) with the possible migration of adult fish following the west coast in the North-East Atlantic, between Celtic and Northern North Sea. Finally, parasites and morphometric analysis were congruent in depicting the existence of sub-structured areas of horse-mackerel in the Mediterranean Sea (western, central and eastern) (Abaunza *et al.*, 2008; MacKenzie *et al.*, 2008; Mattiucci *et al.*, 2008; Murta *et al.*, 2008) (Fig.1a).

The Generalized Procrustes rotation (PR) comparing different ordinations of the horse mackerel populations based on combined molecular and parasitic burden data sets showed slightly significant correlation (Procrustes  $r = 0.39$ ,  $P = 0.05$ ) between the parasitic burden and molecular traits, as shown by the clustering of sampled specimens corresponding to the Mediterranean and Atlantic horse-mackerel (Fig. 2c). Indeed, fish-host populations genetic data accounted only about the 37% of total ordination, whilst the differences in the parasitic burden by larval *Anisakis* spp. for about 95%. This finding further supports the idea that parasites are structuring fish host populations according to regional and temporal scale level. Indeed, the latitude patterns of the



larval *Anisakis* spp. distribution delineated the horse-mackerel putative stocks in the Mediterranean and Atlantic waters according to the different regions of basin waters inhabited by different fish host meta-populations of *T. trachurus*.

#### *Large pelagic fish - The case of the swordfish, Xiphias gladius*

The correct identification of swordfish stocks' is a "hot topic" in fisheries science, being the rationale for the assessment and management of this important resource. As a consequence, different genetic/molecular techniques have been fruitfully used to study the stock structure of this cosmopolitan large pelagic fish that is characterized by a philopatric behavior and a limited gene exchange between different portions of its range, in contrast with its remarkable migration ability. As a consequence of these characteristics, different stocks of swordfish corresponding to many reproductive areas are now recognized. In the Pacific Ocean, swordfish from north-eastern, north-central, south-eastern and south-western areas resulted genetically differentiated (Alvarado-Bremer *et al.*, 2006). In the Atlantic Ocean, a "northern" and a "southern" Atlantic stock are now recognized, with their boundary still questioned based on genetic data: some markers are confirming the 5°N position officially set by ICCAT (International Commission for the Conservation of Atlantic Tunas) (Kasapidis *et al.*, 2006), other data move it northward (Chow *et al.*, 2007) or evidence an intergradation zone in the equatorial area (Garcia *et al.*, 2011) (Fig. 3a). As to the Mediterranean population, it is considered as a further stock, highly isolated and divergent from the Atlantic one and characterized by small population size and low genetic variability (Smith and Alvarado-Bremer, 2010; Chow *et al.* 1997; Chow and Takeyama 2000; Cimmaruta *et al.*, 1998; Kotoulas *et al.*, 1995). These features are explained by the history of this population, which was likely isolated and restricted in the eastern Mediterranean basin during the last two glacial periods and is still poorly exchanging genes with the Atlantic populations (Alvarado-Bremer *et al.*, 2005b). Indeed, Mediterranean swordfish are known to move to the

Atlantic waters out the Gibraltar Straits for feeding, as well as Atlantic specimens may enter the Mediterranean Sea. However, the spawning grounds of the Mediterranean and Atlantic populations, and thus, their gene pools, remain substantially separated due to the philopatric behaviour (Magoulas *et al.*, 1992; Chow *et al.*, 1997). In the Mediterranean Sea waters, a genetic sub-structuring of *X. gladius* has been revealed by phylogeographic analysis of mitochondrial control gene supporting the existence of a “eastern” and a “western” swordfish subpopulation units (Viñas *et al.*, 2010).

The phylogeography and the behavior of swordfish account for by its well defined pattern of genetic variation, making the distinct populations/stocks well identifiable by using genetic/molecular markers on the fish species. Accordingly, a population genetic analysis based on allozyme loci of different swordfish populations from Mediterranean and Atlantic waters (clearly distinguished the Mediterranean stock as distinct from the Atlantic one, as summarized by a Principal Component Analysis (Fig. 3b data from Cimmaruta *et al.*, 1998 and unpublished). The first axis of PCA alone explained 96% of total ordination (another 2% was explained by the second axis) illustrating that for this fish species even conservative markers, such as allozymes, have a high resolution power.

The North Atlantic swordfish recently benefitted of the management actions that rebuilt its stock consistency (Nielson *et al.*, 2013). On the contrary, the Mediterranean swordfish is still in need of an effective management to counteract the loss of its spawning stock biomass and the ever-increasing percentage of juveniles in its landings, signalling a possible stock collapse into the next generation time of 7-10 years (Tserpes *et al.*, 2011).

According to the results achieved on the different species of the larval *Anisakis* recognised in *X. gladius* from the Mediterranean Sea and Atlantic Ocean fishing grounds, different populations of swordfish have been distinguished from the two basin waters (Garcia *et al.*, 2011; Mattiucci *et al.*, submitted). Indeed, among all the metazoan taxa of *X. gladius* identified in several samples of

the fish species captured in Mediterranean Sea and Atlantic Ocean waters, the larval stages of *Anisakis* were among those mainly responsible accounting for by the differences detected between the Mediterranean and Atlantic populations (Mattiucci *et al.*, submitted). In fact, *A. pegreffii* was identified only in *X. gladius* from the Mediterranean Sea fishing grounds; whereas, the larval species of *A. brevispiculata*, *A. paggiae*, *Anisakis* sp. 2, and *Anisakis simplex* (s.s.), were absent in *X. gladius* sampled from the same Mediterranean waters (Fig. 3a) *A. pegreffii* was also found in co-infection with *A. physeteris* genetically recognised in the samples from the central and the southern Tyrrhenian Sea. However, the significant different infection pattern with *Anisakis pegreffii* and the absence of *A. physeteris* in the swordfish sampled from the Ionian Sea with respect to the central and Southern Tyrrhenian Sea (Fig. 3a) suggests a further investigation of swordfish from other parts of the eastern Mediterranean Sea, searching for a possible accordance as suggested by genetic/molecular data (Viñas *et al.*, 2010). On the other hand, *A. physeteris* was found at high proportion level and showed higher infection levels in *X. gladius* fished in east-tropical and the tropical-equatorial Atlantic waters, with respect to those observed in the Mediterranean samples (Garcia *et al.*, 2011; Mattiucci *et al.*, submitted). Indeed, in the Atlantic swordfish, the species *A. physeteris*, *A. brevispiculata*, *A. paggiae* and *Anisakis* sp. 2 are the dominant taxa. These findings are in accordance with the geographical distribution reported so far for those species (Fig. 3 b). The PCA analysis based on the distribution pattern and infection levels with *Anisakis* spp. from swordfish depicted the Mediterranean samples clustering separately from the Atlantic ones (Fig. 3c). In addition, in the Atlantic waters, a cluster formed by the “northern” sample distinct from a cluster including the “southern” swordfish was observed (Mattiucci *et al.*, submitted) (Fig. 3c).

The use of *Anisakis* spp. as biomarkers agrees with the genetic results gathered on the fish host when describing the existence of a Mediterranean Sea stock and two discrete Atlantic stocks, i.e. a “northern” and a “southern” Atlantic stock of the fish species (Fig. 3c).

However, when defining the boundary between the two Atlantic stocks, the results obtained by different genetic/molecular markers on *X. gladius* are discordant each other, either confirming the 5°N position, or moving it northern to 5°N, or evidencing an intergradation zone in the equatorial area. According to the *Anisakis* spp used as markers, the distinction between the NW swordfish stock from the remaining Atlantic populations is in agreement with the bulk of genetic data; while, the boundary between the NW and the “southern” stock seems to be less certain. This suggests a possible boundary between the northern and southern stocks, northern to 5°N, or at least, the existence of a mixing area in the tropical-equatorial Atlantic waters (Garcia *et al.*, 2011; Mattiucci *et al.*, submitted) (Fig. 3a).

## DISCUSSION AND FUTURE PERSPECTIVES

A high genetic homogeneity of marine species has been postulated for years, based on the idea that in the marine environment there were few physical barriers limiting gene flow between fish populations (Palumbi, 1994). However, in the last decade increasing data have shown that marine species may be spatially structured even on a very small scale due to a number of factors ranging from local self-recruitment to historical vicariance (Cowen *et al.*, 2000; 2006). Also, the marine environment resulted to be provided of geographic barriers, habitat discontinuities and transition zones, able to exert different selective pressures on geographically discrete populations of marine species (Cimmaruta *et al.*, 2005; Lemaire *et al.*, 2005; Milano *et al.*, 2014; Riginos *et al.*, 2001; Sà-Pinto *et al.*, 2012). Historical and oceanographic characteristic of the Mediterranean-Atlantic transition are among the main factors in designated distinctive and geographical separated stocks of several fish species, as shown by an increasing number of investigations carried out at both large and fine geographic scale and providing phylogeographical scenarios for several fish species from these basins. Surveys on the genetic structure of fish populations over a large geographic range, spanning the North-East Atlantic Ocean, the Mediterranean Sea (west and

east) and the Black Seas, regarded many fish species, including among others *Engraulis encrasicolus* (Magoulas et al., 2006), sparids species (Bargelloni et al., 2003; 2005), the anglerfish *Lophius budegassa* and *L. piscatorius* (Charrier et al., 2006), the Atherinidae *Atherina* spp. (Pujolar et al., 2012), and the mackerels *Scomber scombrus* and *S. japonicus colias* (Zardoya et al., 2004). In spite the high number of studies, very few contributions report of a multidisciplinary approach for the fish stock assessment in Mediterranean versus Atlantic waters, which includes either genetic data, biological (parasites) and morphological survey of the fish host. To date, the only representative cases were those concerning fish stock assessment of Horse mackerel and European hake, performed in the framework of the European Projects HOMSIIR (Abaunza et al., 2008) and GENHAKE (Nascetti et al., 2000), respectively. Some other Authors have used a meta-analysis approach for biological (not including parasites) and genetic data sets acquired in various fish species to provide an integrated picture of Mediterranean-Atlantic population structure and phylogeography (Patarnello et al., 2007), also in the context of global patterns (Galarza et al., 2009; Riginos et al., 2011). Their findings evidenced an overall poor ability of biological factors as predictors of population/stock genetic structure (Galarza et al., 2009) or failed in showing any particular association between biological traits and genetic variables (Patarnello et al., 2007) but all highlighted the role of biogeographic-environmental factors in shaping the genetic structure of fish species.

In the present review we showed that *Anisakis* parasites used as biological tags do provide a biological data set able to identify fish stock structure generally in agreement with the findings of molecular approaches. The meta-analysis approach performed by generalised Procruster Rotation analysis (PR) on the genetic (allozyme markers) and biological (*Anisakis* spp. parasites) data sets acquired from the same fish populations of the Mediterranean Sea and Atlantic Ocean, in the framework of a multidisciplinary approach, produced a significative association between parasites as biomarkers and the genetic variation detected at the allozyme level and agreed in delineating

the stock structure of the demersal and pelagic fish studied (Fig.1c, Fig. 2c). The results obtained also suggest that the combined data sets produce stronger information on the population structure of a fish species rather than that indicated by a single data set (Fig. 2c). This is due to the different features of the two kinds of data, where the parasites (biomarkers) provide knowledge the movements of fish specimens for feeding purposes during their life span, while the fish population genetic data (molecular/genetic markers) those for reproduction averaged over many generations. Thus, genetic studies on fish host populations can detect changes over an evolutionary time scale. These differences may cause incongruences in the results achieved by means of the two different methodological approaches, as shown for example in the case of *T. trachurus* stock assessment, where fish genetic data accounted a less structuring power with respect to the parasites which, on the contrary, accounted most of the differences found at the meta-population level. On the other hand, parasites as biomarkers are generally more concordant with the biological and life-history traits of the fish host, as demonstrated in the case of *T. trachurus* (Abaunza *et al.*, 2008a) and *M. merluccius*. The occurrence and composition of the parasite fauna in fish populations, as other fish biological parameters, are very good and suitable biomarkers when considering a fish stock in a temporal (as related to the fish life-history) and spatial (as related to the geographic region) scale, because they reflect the environmental variables in which the fish species is spending its life-history. Thus, their potential application as biomarkers ranges from providing knowledge of the tracing fish migrations across different marine ecosystems to the identification of the marine grounds origin for a given fish species.

This, in turn, means that the phenotypic markers (including parasites) of a fish species have greater application in studies on the short-term variation caused by the environment and other abiotic and biotic factors of a marine ecosystem (Begg and Waldmann, 1999). Therefore, comparison of incongruences between genetic and parasitological patterns provides information on the nature of fish stocks and on the time scale involved in their characterization. Such kind of

information can be obtained only within the frame of a multidisciplinary approach to fish stock assessment, proofing for the high power of this approach.

Based on their features as biological tags, parasites can be used ecosystems as good indicators of water regions inhabited by the fish species. Indeed, the parasites and their density in a region could reflect the temporal and spatial population dynamics of the fish species in a given geographical region. However, in turn, the demography and population dynamics of definitive and intermediate host populations could influence the demography and density of a given host-specific parasite, as well (Mattiucci and Nascetti, 2007, 2008). Thus, monitoring a fish subpopulation or stock at regional scale level, including its specific parasite fauna and their abundance levels as biomarkers, could have implications also in fish management and conservation measures.

In addition, future comparative phylogeographic studies between parasites and their hosts maybe useful in determining fish host populations over different geographical areas. Indeed, parasite phylogeography could provide information to infer host population structure. The relative mutation rates of homologous loci between parasites DNA and that of their hosts suggests that some parasites have a faster generation time than hosts (Whitman and Parker, 2005). This could lead to a faster accumulation of genetic variation in the parasite genome allowing to infer host population structure from the parasite population structure. In other words, the divergence time separating parasite populations could be shorter than that recorded in host population. Different inferences could be acquired by the different genetic markers used, and this occurs both for the fish hosts and parasite species. Mitochondrial DNA genes, due to the faster accumulation of base pairs substitution and maternal inheritance, are suitable genetic markers to infer population structure in both fish and parasite species. For instance, Criscione *et al.* (2006) indicated that parasite trematode genotypes are more sensible to indicate the natal stream of an individual of *Oncorhynchus mykiss*, rather than the host genotypes. Similarly, Baldwin *et al.* (2011) used

population genetic structure based on the cytochrome c oxidase 2 (*cox2*) mitochondrial DNA gene of larval nematodes of the genus *Anisakis* to infer the stock structure of Pacific sardine (*Sardinops sagax*) in the California current. Mitochondrial haplotype distribution of larval *Anisakis* spp. obtained by those Authors, from both parsimony networks and analyses of molecular variance, revealed a panmictic distribution of these parasites, which infects sardines throughout the California current ecosystem. Based on the results achieved, Baldwin *et al.* (2011) hypothesized that the panmictic distribution of the larval *Anisakis* spp. populations may be a result of the presumed migratory pathways of the intermediate host (the Pacific sardine), moving into the northern portion of the California current in summer and returning to the southern portion to overwinter and spawn in spring. However, the wider geographic range of paratenic (large predatory fish), and final (cetaceans) hosts has been also postulated to explain the observed distribution pattern.

On the other hand, other mitochondrial markers, such as the mtDNA *cox1* has been suggested (Cross *et al.*, 2007) as suitable indicator of nematode population structure of *Anisakis simplex* (*s.s.*), potentially providing knowledge about its fish host, the Atlantic herring, *Clupea harengus* (Cross *et al.*, 2007). This study has indicated that mtDNA *cox1* gene sequences of *A. simplex* (*s.s.*) analysed between and within spawning seasons of *C. harengus* showed a high degree of temporal stability, highlighting the potential suitability of this molecular marker in this parasite species as biological tag of fish-host stocks (Cross *et al.*, 2007).

Future studies on the phylogeographical analysis including population genetic structure of parasites as biomarkers and that of their host evolutionary units, inferred from highly polymorphic genes (such as mitochondrial DNA and or DNA microsatellites) could represent a further tool to be included in a holistic approach to infer stocks structure of fish species over its range of distribution.



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## LEGENDS TO THE FIGURES

**Figure 1.** - The case study of stock identification of a demersal fish species, the European hake *Merluccius merluccius*, from the Mediterranean Sea and Atlantic Ocean, as inferred from biological data, biomarkers (*Anisakis* spp. parasites) and the population genetics data (allozymes) of the fish populations (data from Nascetti et al. 2000; Mattiucci et al. 2004; Cimmaruta et al. 2005). (A) Relative proportions of the *Anisakis* spp. larvae genetically identified ( $N_{\text{parasites}} = 1950$ ) in *M. merluccius* from the sampling localities from the Mediterranean Sea and the Atlantic Ocean (data from Mattiucci et al. 2004), plotted with the stock units as identified by the biological and morphometric analyses (data from Nascetti et al. 2000). A different colour identifies a putative stock unit as defined by morphometric datasets of the fish host. Dotted lines identify the

boundaries between hake stock units, as identified by genetic/molecular markers; (B) Principal Component Analysis (PCA) inferred from the larval *Anisakis* spp. bio-markers, identified genetically in the European hake specimens (*M. merluccius* sampled in the Mediterranean (white circles) and Atlantic (black circles) basins. The first axis of PCA explains about 60% of total ordination, and PCA 2 about 30%; (C) A generalized Procrustes rotation (PR) performed with R (R Development Core Team, 2012) was used to compare different ordinations of the European hakes *M. merluccius* ( $N_{\text{fish}} = 1306$ ), based on combined molecular and parasite datasets. The latter was computed by considering the number ( $N_{\text{parasites}} = 1950$ ) of larval parasites belonging to *A. pegreffii*, *A. simplex* (s. s.), *A. physeteris*, *A. typica*, *A. ziphidarum* and *A. brevispiculata*. A Procrustes test (also known as analysis of congruence) with  $n = 1000$  bootstrap replicates was used to estimate the significances of the Procrustes statistics, assessing similarities between different ordinations, and the Procrustes correlation  $r$  was derived from the symmetric Procrustes residual (Peres-Neto and Jackson, 2001). Analysis showed a significant correlation (Procrustes  $r = 0.59$ ,  $P < 0.01$ ) between the *Anisakis* spp. burden and molecular traits, showing the clustering of sampled specimens in two well-defined clusters corresponding to the Mediterranean (white circles) and Atlantic stocks (black circles). Code numbers and coloured ellipses are related to the sampling localities, as appearing in Fig. 1A

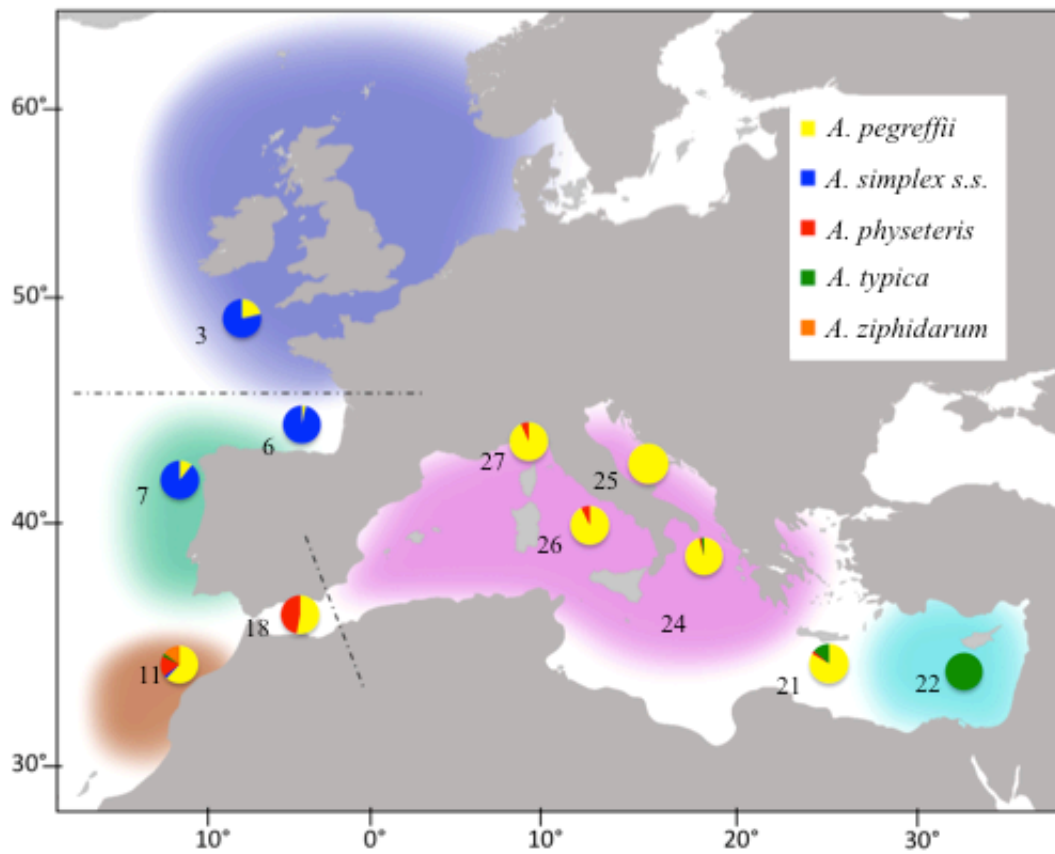
**Figure 2.** The case study of stock assessment of a small pelagic fish species, the horse mackerel *Trachurus trachurus*, from the Mediterranean Sea and Atlantic Ocean waters, as inferred from biological data, biomarkers (parasites), and the population genetics dataset (allozymes) of the fish populations (data from Abaunza et al. 2008a; Cimmaruta et al. 2008; Mattiucci et al. 2008; Murta et al. 2008). (A) Relative proportions of the *Anisakis* spp. identified genetically ( $N_{\text{parasites}} = 2200$ ) in *T. trachurus* from the sampling localities from the Mediterranean Sea and the Atlantic Ocean (data from Mattiucci et al. 2008), plotted with the stock units identified by the biological and



morphometric analyses (data from Murta et al. 2008). Different colours identify each putative stock unit as defined by morphometric datasets of the fish host; (B) Principal Component Analysis (PCA) inferred from larval *Anisakis* spp. as biomarkers, identified genetically in the horse-mackerel specimens (*T. trachurus*) sampled in the Mediterranean (white circles) and Atlantic (black circles). The first axis of PCA explains about 92% of total ordination, whilst PCA 2 about 7%; (C) A generalized Procrustes rotation (PR), performed with R (R Development Core Team, 2012) used to compare different ordinations of the Atlantic horse mackerels (*T. trachurus*) analysed ( $N_{\text{fish}} = 2241$ ), based on combined molecular and *Anisakis* spp. burden distance matrices. The latter was computed by considering the number of individual parasites ( $N_{\text{parasites}} = 2200$ ) (fourth-root transformed to improve normality and remove the mean/variance relationship) belonging to *A. pegreffii*, *A. simplex* (s.s.), *A. physeteris* and *A. typica*. A Procrustes test (also known as analysis of congruence) with  $n = 1000$  bootstrap replicates was used to estimate the significance of the Procrustes statistics, assessing similarities between different ordinations, and the Procrustes correlation  $r$  was derived from the symmetric Procrustes residual (Peres-Neto and Jackson, 2001). Analysis showed significant correlation (Procrustes  $r = 0.39$ ,  $P = 0.05$ ) between the parasitic burden and molecular traits, as shown by the clustering of sampled specimens corresponding to the Mediterranean (white circles) and Atlantic stocks (black circles). Molecular distance between specimens accounted for about 37% of total ordination, whilst the distance based on the parasitic burden with *Anisakis* for about 95%. Code numbers and coloured ellipses are related to the sampling localities, as appearing in Fig. 2A

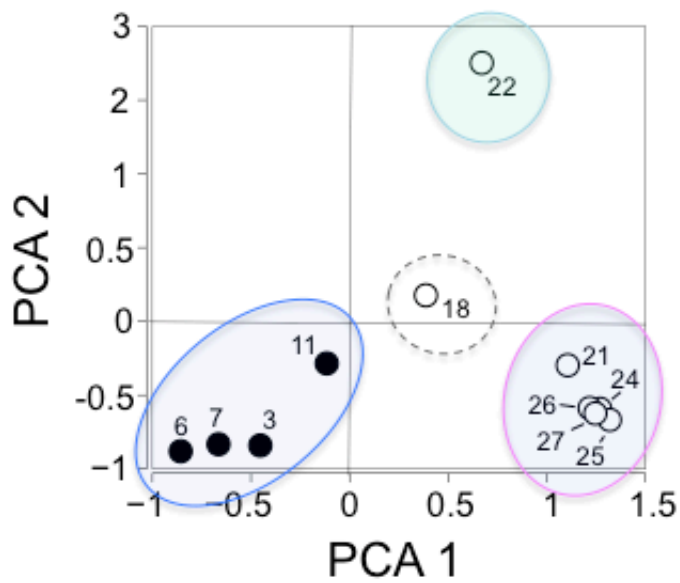
**Figure 3.** The case study of stock assessment of a large pelagic fish species, the swordfish *Xiphias gladius* from the Mediterranean Sea and Atlantic Ocean, as inferred from biological data, biomarkers (parasites) and the genetic datasets (allozymes) of the same fish populations (data from Garcia et al. 2011; Mattiucci, unpublished data.). (A) Relative proportions of the *Anisakis*

spp. identified genetically ( $N_{\text{parasites}} = 405$ ) in *X. gladius* from the sampling localities from the Mediterranean Sea and the Atlantic Ocean (data from Garcia et al. 2011; Mattiucci, unpublished data), plotted with the stock units as suggested by the genetic/molecular results (Kotoulas et al. 1995; Alvarado-Bremer et al. 2005a, b; Chow et al. 2007). Dotted lines identify the boundaries between swordfish stock units; (B) Principal Component Analysis (PCA) inferred from the parasitic burden of the larval *Anisakis* spp. identified genetically and used as biomarkers, in the swordfish *X. gladius* specimens sampled in the Mediterranean (white circles) and Atlantic (black circles) basins. PCA analysis was computed by considering the number of individual parasites (fourth-root transformed to improve normality and remove the mean/variance relationship) belonging to *A. pegreffii*, *A. simplex* (s. s.), *A. physeteris* and *A. brevispiculata*. The first axis of PCA explains about 54% of total ordination, and PCA 2 about 22%; (C) Principal Component Analysis (PCA) inferred from Nei's distance matrix based on allozyme data (source: Cimmaruta et al. 1998) performed on swordfish specimens analysed genetically ( $N_{\text{fish}} = 323$ ). The first and second axis accounted for 96 and 2% of total ordination, respectively.

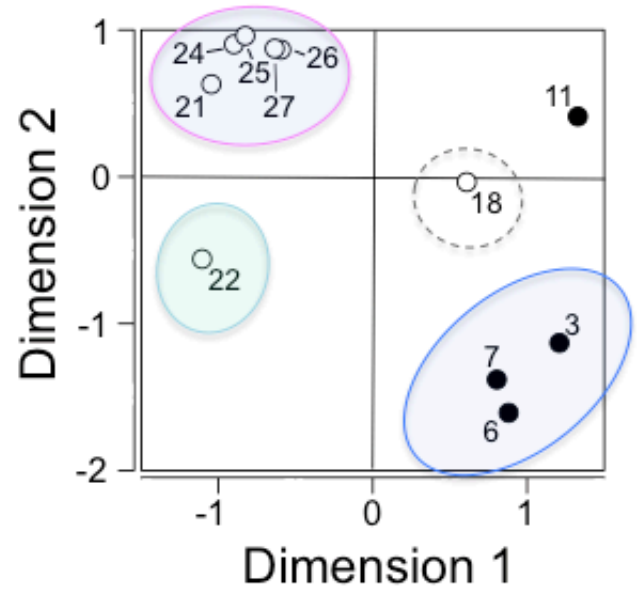


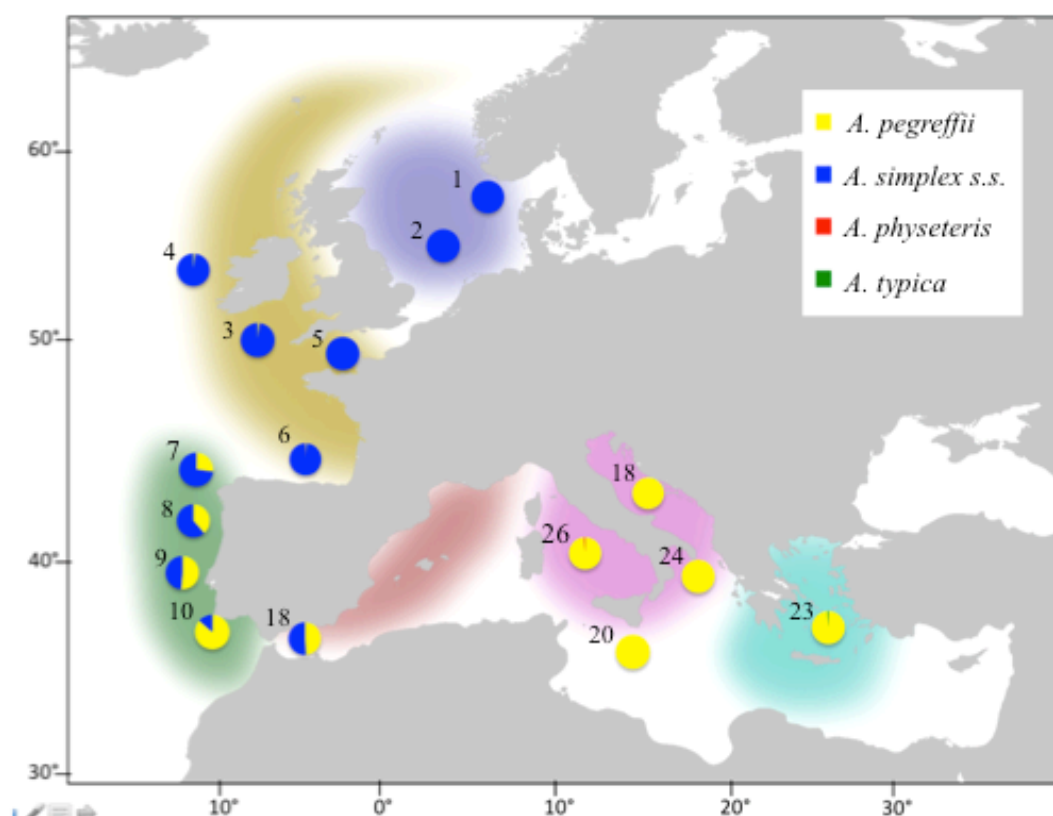
Atlantic Ocean	
3	Great Sole Bank
6	Bay of Biscay
7	Galician Coast
11	Atlantic coast of Morocco
Mediterranean Sea	
18	Alboran Sea
21	Crete Sea
22	Off Cyprus
24	Ionian Sea
25	Adriatic Sea
26	Central Tyrrhenian Sea
27	Ligurian Sea

PCA parasites

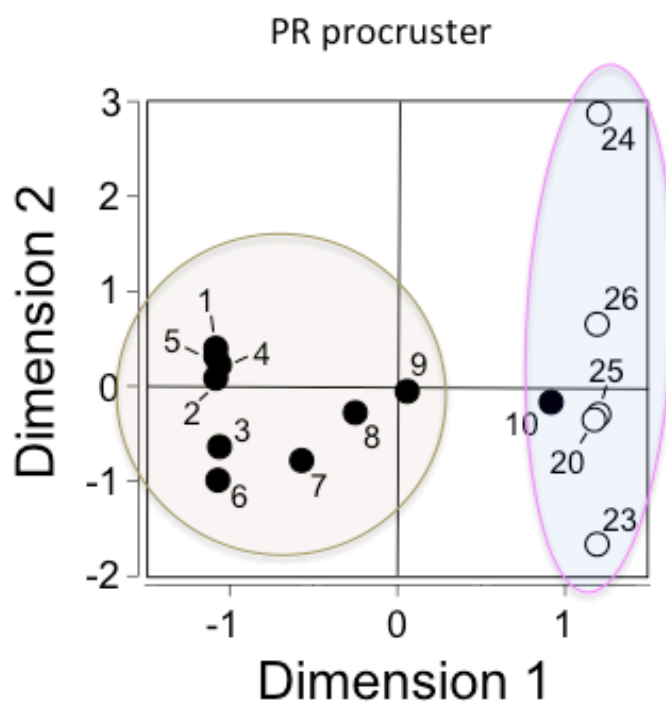
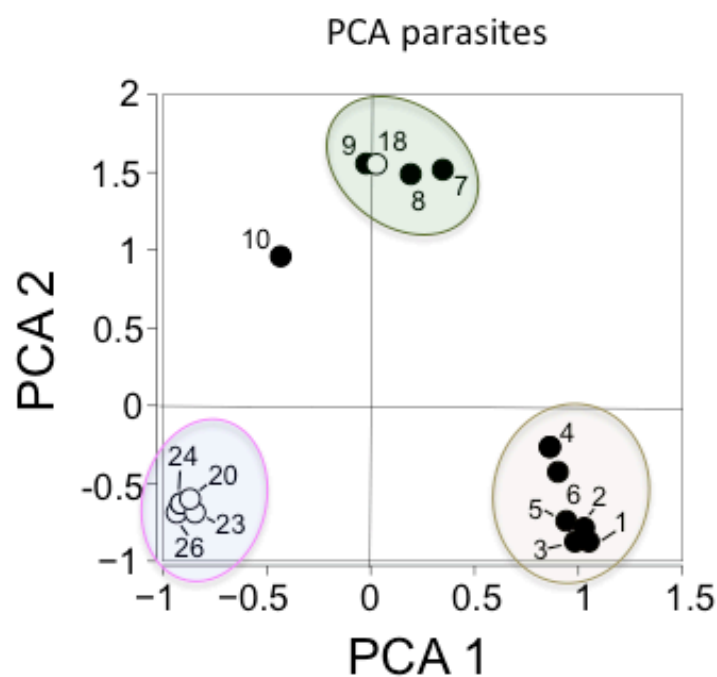


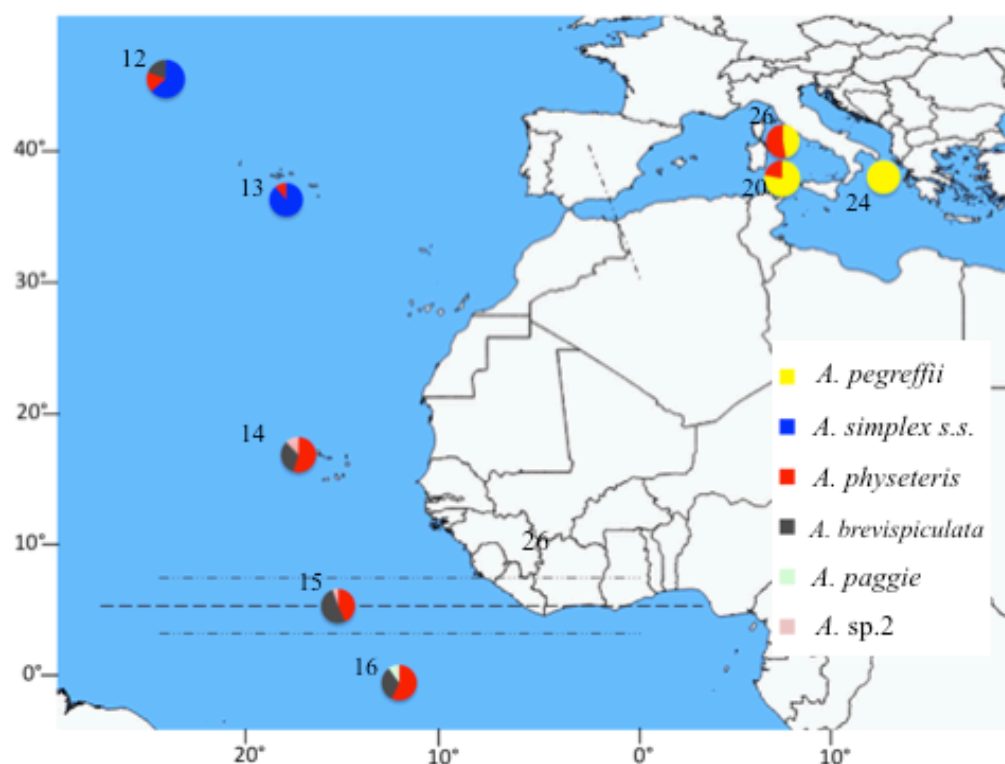
PR procruster





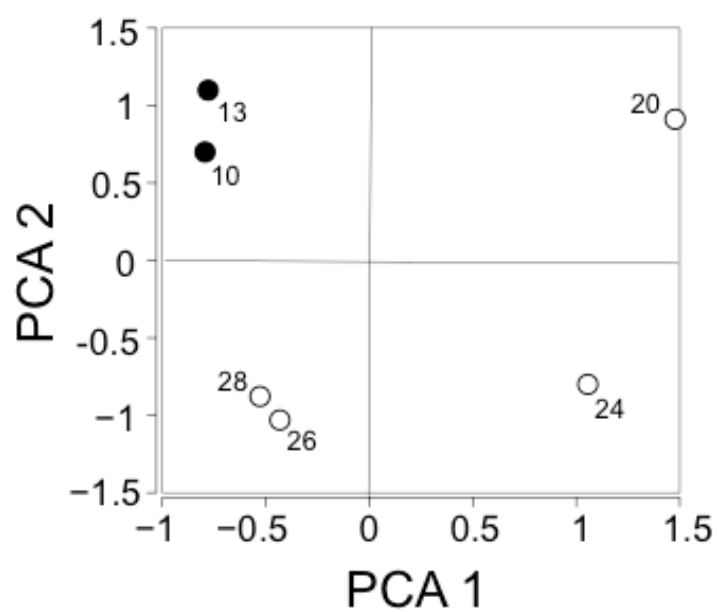
Atlantic Ocean	
1	South of Norway
2	North sea
3	Great Sole Bank
4	West Ireland
5	West English Channel
6	Bay of Biscay
7	Galician Coast
8	North Portugal
9	Central Portugal
10	South Portugal
Mediterranean Sea	
18	Alboran Sea
20	South of Sicily
23	Aegean Sea
24	Ionian Sea
25	Adriatic Sea
26	Central Thyrrhenian Sea





Atlantic Ocean	
10	South Portugal
12	North West Atlantic
13	Central North Atlantic
14	Eastern Tropical Atlantic
15	Tropical Equator Atlantic
16	Central South Atlantic
Mediterranean Sea	
20	South of Sicily
24	Ionian Sea
26	Central Thyrrhenian Sea
28	South <u>Thyrrhenian</u> Sea

PCA molecular



PCA parasites

