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Parasites of the Blackspot Seabream, *Pagellus bogaraveo*, as Biological Tags for Stock Identification

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ABSTRACT

The blackspot seabream, *Pagellus bogaraveo* (Brünnich, 1768), is an important sparid fish targeted mostly by Portuguese and Spanish fisheries in the north-east Atlantic. This is the first comprehensive study of its parasite fauna, as well as the first attempt to apply parasite tags to the identification of *P. bogaraveo* stocks. In this study, 348 specimens of *P. bogaraveo* from four localities in mainland Portuguese waters (Matosinhos, Figueira da Foz, Peniche, and Sagres), and the two Atlantic archipelagos of Madeira and the Azores, were observed for the presence of metazoan parasites. Parasites were identified to the lowest possible taxonomic level, and infection levels were determined. Anisakid nematodes were identified using PCR-RFLP and sequencing of the ITS region. Ectoparasite communities, as well as gastrointestinal helminth communities, were the object of in-depth analysis. A total of thirty-seven parasite taxa were detected, including twenty-four new records in this host. Biogeographical differences in parasite assemblages were detected at the regional level. Following established criteria for the adequacy of parasites as biological tags for the study of fish stocks, 5 parasite taxa were selected as biological tags of *P. bogaraveo* in the north-east Atlantic. These species indicate the existence of three distinct blackspot seabream stocks: one in the Portuguese continental shelf/slope (ICES area IXa), another in the Azores (ICES area X), and another in Madeira (sub-area 1.2 of FAO area 34).

SUMÁRIO

O goraz, *Pagellus bogaraveo* (Brünnich, 1768), é um peixe da família Sparidae, economicamente importante e que é alvo de pesca no Atlântico nordeste, principalmente por parte de Portugal e Espanha. Este é o primeiro estudo abrangente sobre a parasitofauna desta espécie, bem como a primeira tentativa de utilização dos parasitas como marcadores biológicos para a identificação de stocks de goraz. Neste estudo, 348 exemplares de *P. bogaraveo* provenientes de 4 localidades ao longo da costa continental Portuguesa (Matosinhos, Figueira da Foz, Peniche e Sagres), e ainda dos arquipélagos da Madeira e dos Açores, foram analisados quanto à presença de parasitas metazoários. Os parasitas foram identificados até ao mais baixo nível taxonómico possível, tendo sido determinados os níveis de infecção. Os nemátodes anisaquídeos foram identificados através de PCR-RFLP e sequenciação da região ITS. Foram realizados estudos aprofundados sobre as comunidades de ectoparasitas e de parasitas gastrointestinais. Foi encontrado um total de trinta e sete taxa, tendo vinte e quatro destes sido observados neste hospedeiro pela primeira vez. Verificou se a ocorrência de diferenças biogeográficas nas comunidades de parasitas a nível regional. Cinco taxa foram seleccionadas como marcadores biológicos para a identificação de stocks de *P. bogaraveo* no Atlântico nordeste, seguindo critérios já estabelecidos. Estas espécies indicam a existência de três stocks distintos de goraz: um em águas continentais portuguesas (ICES área IXa), um nos Açores (ICES área X), e outro na Madeira (sub-área 1.2 da FAO área 34).

RÉSUMÉ

La dorade rose, *Pagellus bogaraveo* (Brünnich, 1768), est un poisson de la famille Sparidae, économiquement important et qui est objet de pêche dans l'Atlantique nord-est, en particulier au Portugal et en Espagne. Ce travail s'agit de la première étude exhaustive sur la parasite-faune de cette espèce, aussi bien que la première tentative d'utilisation des parasites comme bio-marqueurs pour l'identification des stocks de dorades rose. Dans cette étude, 348 exemplaires de *P. bogaraveo* provenants de 4 localités ao long de la côte continentale portugaise (Matosinhos, Figueira da Foz, Peniche et Sagres), et encore des archipels de Madeira e Açores, ont été analysés quant à la présence des parasites métazoaires. Les parasites ont été identifiés au plus bas niveau taxonomique possible, étant été déterminés les niveaux d'infection. Les nématodes anisaquides ont été identifiés a travers de PCR-RFLP et séquençage de la région ITS. Des études approfondis sur les communautés d'ectoparasites et de parasites gastro-intestinales ont été réalisés. Un total de trente sept taxa a été trouvé, parmi lesquels vingt quatre ont été retrouvés dans cet hôte pour la première foi. On a vérifié l'occurrence de différences biogéographiques dans les communautés de parasites au niveau régional. Cinq taxa ont été sélectionnés comme bio-marqueurs pour l'identification des stocks de *P. bogaraveo* dans l'Atlantique nord-est, selon les critères établis. Ces espèces indiquent l'existence de trois stocks distincts de dorade rose: l'un dans les eaux continentales portugaises (ICES area IXa), l'un dans les Açores (ICES area X) et un autre à Madeira (sub-area 1.2 da FAO area 34).

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CHAPTER 1

General Introduction and Aims

THE BLACKSPOT SEABREAM, *Pagellus bogaraveo*

Distribution, ecology and reproduction

The blackspot or red seabream, *Pagellus bogaraveo* (Brünnich, 1768) is a benthopelagic fish that occurs in the Mediterranean and in the Northeast Atlantic Ocean from Norway to Mauritania, Madeira and the Canary Islands, at depths of up to 800 m (Spedicato et al. 2002). It is a sparid fish characterized by a reddish hue and a distinctive black spot on either side at the origin of the lateral line (Fig. 1). Blackspot seabream exhibit ontogenetic habitat changes, with juveniles preferring shallow waters on the continental shelf or around islands, and pre-adult and adult individuals inhabiting deeper waters, mostly on the continental shelf break and slope and over steep sites around islands and seamounts in oceanic environments (Morato et al. 2001; Menezes et al. 2006).

The blackspot seabream is an omnivore with a preference for animal prey (Stergiou & Karpouzi, 2002). Its diet comprises fish (especially Myctophidae) and several groups of invertebrates, the most prominent of these being Thaliacea and Ophiurida, but also molluscs, crustaceans, annelids, siphonophores, hydrozoans and sponges, among others; it will also on occasion consume algae (Morato et al. 2001). It can feed on benthic, benthopelagic and pelagic species, and is known to display vertical migrations. Diet composition is relatively similar in different size groups, although there appears to be a preference for larger prey with increased size (Morato et al, 2001).

Pagellus bogaraveo is a protandrous hermaphrodite, with most individuals maturing first as males, and later as females (Micale et al. 2002, 2011). In the wild, size at maturity has been estimated at 30,1 cm for males and 35,1 cm for females in the Strait of Gibraltar (Gil & Sobrino 2001) and 26,2 cm (age 3) and 29,2 cm (age 4), respectively, in the Azores (Estácio et al. 2001). Micale et al. (2002) suggest that sex inversion may be directly related to fish age rather than size. Other authors suggest that a density-dependent regulation of the onset of maturity and/or sex change may also occur in this species (Lorance 2011). Either way, size and age at maturity seems to be very variable, and some males may never change sex (Krug 1990). There is also a high incidence of gonochoric females, both in the wild and in captivity (Krug 1990; Micale et al. 2002, 2011). Interestingly, Micale et al. (2002) have observed that the proportion of male and female tissue within the ovotestis is not always an indicator of functional sex.

Vulnerability to fishing pressure

The sequential hermaphroditism exhibited by *P. bogaraveo* is a reproductive strategy that enables individuals to maximize lifetime reproductive success when there is differential size-dependent mating success for each sex (Buxton & Garratt 1990). However, for a protandrous hermaphrodite this means that larger fish will be mostly female, and since these are the main targets of commercial fisheries, fishing pressure will disproportionately target females, thus severely reducing spawning biomass (Erzini et al. 2006). This, along with other features such as a relatively long life-span and slow maturation, which are common to many deep-water species, render this fish particularly vulnerable to overexploitation.

Recent studies have found dramatic decreases in blackspot seabream mean length in some regions, such as the Strait of Gibraltar, as a result of fishing mortality (Erzini et al. 2006). Decrease in spawning biomass and recruitment failure due to overfishing of large, mature females is thought to have caused the collapse of the previously abundant *P. bogaraveo* stock of the Cantabrian Sea (Erzini et al. 2006; Lorance 2011).

Furthermore, a tendency for earlier maturation has also been observed (Estácio et al. 2001), which is usually a response to high fishing pressure. Fish stocks respond to selective removal of larger individuals by evolving towards earlier maturation, either by phenotypic plasticity, or genetically through fisheries-induced evolution, in which case the changes are usually irreversible and may lead to loss of genetic variability (Kuparinen & Merilä 2007). Since fecundity correlates with body size, lower size at maturity may also lead to poorer reproductive output, further reducing the fitness of the exploited populations (Ratner & Lande 2001).

Current state of fisheries in the north-east Atlantic

The vast majority of captures of *P. bogaraveo* originates from the north-east Atlantic, with minor catches in the Mediterranean and central-eastern Atlantic (Basurco et al. 2011), although it is an important species in some areas of the western Mediterranean (Spedicato et al. 2002). Currently, Portugal and Spain are the main producing countries (Basurco et al. 2011).

The International Council for Exploration of the Seas (ICES) has established three management units for this species in the north-east Atlantic (Fig. 2):

- Areas VI, VII, and VIII;
- Area IX; and
- Area X.

Areas VI, VII and VIII were mostly exploited by France and the UK since the early 20th century, until severe depletion of the targeted stocks. Landings have decreased severely and evidence no signs of recovery (Caddy & Surette 2005, ICES 2008, Lorance 2011).

Area IX includes all of the mainland Portuguese coast, plus Galicia in the north and the Huelva coast down to the Gibraltar Strait, where there is a significant Spanish fishery. In recent years, after warning signs such as the decline of catches and decrease in mean length, regulations have been imposed on the Spanish fleet operating in the area of the Gibraltar Strait, but there is still no evidence of sustainability of this fishery (Herrera 2012). All along the Portuguese coast, *P. bogaraveo* catches have been declining, and in some regions where it used to be abundant, such as Setúbal, the stock is considered, in the words of local fishermen, 'finished'. Reported catches of blackspot seabream in mainland Portugal have been just under 100 tonnes in 2011, of a total fishing quota of 166 t (INE, 2012).

The bulk of blackspot seabream catches is concentrated in ICES area X, which includes the Azores, where *P. bogaraveo* is an important commercial species, with annual catches above 1000 t. This is the only ICES region where catches have remained stable in recent years, and surveys have detected increases in abundance indices.

Madeira is located in sub-area 1.2 of FAO area 34, central-eastern Atlantic (Fig. 2). Fisheries in Madeira are mostly dominated by tunas and black scabbardfish, followed by small pelagic fish. Sparids comprise only a small fraction of the catches, and there is very little information on *P. bogaraveo* stocks in this region.

Aquaculture production

The excellent palatability and high market value of the blackspot seabream make it a prime candidate for aquaculture diversification, which has heretofore been mainly restricted to a few species. The decline of wild populations of *P. bogaraveo,* as well as continued market demand, have made its production more appealing (Basurco et al. 2011). There seem to be no major problems with either larval culture or ongrowing (Peleteiro et al. 2000, Basurco et al. 2011). Blackspot seabream may reach sexual maturity earlier in captivity than in the wild (Micale et al. 2011), possibly due to better nutritional conditions. In addition, spontaneous spawning in tanks has been reported (Peleteiro et al. 2000; Micale et al. 2011), and was also very easily provoked upon simple manipulation of the fish, which is unusual in captive fish and may be an advantage for the production of this species in aquaculture (Micale et al. 2002). However, its slow growth rate, long ongrowing cycle (42 months), and sensitivity to culture conditions (Basurco et al. 2011), have posed some obstacles to its production. Growth performance is considerably better in cages that in land-based tanks (Olmedo et al. 2002).

Commercial production began in Galicia in 2002, using offshore floating net cages, and annual production is increasing (Basurco et al. 2011). As long as quality standards are maintained, mainly in terms of flavour and texture of the flesh, the market seems to be very receptive to this species and, in fact, blackspot seabream produced in Spain are currently being sold in Portugal.

THE STUDY OF FISH STOCKS: BIOLOGY MEETS FISHERIES

Identification methods and the stock concept

The relative merits of the different approaches to stock identification are largely dependent on the operational definition of *stock* (Swain et al. 2005). Numerous definitions of stock have been advanced in fishery science, spanning a continuum from the practical 'fishery stock' concept, which designates a group of fish commercially exploited in a certain area, and is basically a management unit, to the 'genetic stock', which is a reproductively isolated unit, genetically different from other stocks of the same fish species (Carvalho & Hauser 1994). Usually, if not always explicitly, the stock concept incorporates both the idea of a biological population and practical management considerations (Swain et al. 2005).

Genetic analyses have been among the most widely used methods since the relevant technologies were developed. They include allozymes, mitochondrial DNA, repetitive nuclear DNA such as microsatellites, and Random Amplified Polymorphic DNA (RAPD), among other techniques (Cadrin et al. 2005). Most genetic methods are based on neutral markers, stretches of non-coding DNA that can be assumed to diverge at relatively constant rates over time in reproductively isolated populations. However, complete reproductive isolation of fish populations in the marine environment is unlikely, and some studies have shown that, for the sample sizes generally used in stock identification studies, even a small number of migrant fish per generation is sufficient to prevent detectable heterogeneity in neutral genetic markers (Carvalho & Hauser 1994). The reproductive biology of fish, and in particular the phenomenon of sequential hermaphroditism that occurs in *P. bogaraveo* and other species, introduces a degree of complexity into genetic studies using mitochondrial DNA, which is exclusively maternally transmitted, but can potentially be transmitted by all individuals in the population in sex-changing fish. The consequences of this uncommon reproductive system for the genetic structure of fish populations are still poorly understood (Chopelet et al. 2009).

The study of morphological analyses, such as morphometrics and meristics, and of life history parameters, such as growth, reproduction, and recruitment, can overcome some of the problems of neutral genetic markers because the features under study are often related to fitness and respond to selection, which can be much quicker than neutral evolution, even in the absence of reproductive isolation (see Swain et al. 2005). However, it is often difficult, if not impossible, to disentangle genetic differences from phenotypic plasticity on features such as growth, age and size at maturity, and even morphological characters (Thompson 1991).

Yet the fact that some markers are partly or exclusively environmental in nature does not detract from their relevance and utility in stock identification (Waldman 2005). In fact, in many cases environmental markers can be more informative than genetic markers, for example when reproductive isolation between stocks is absent or incomplete, and/or when there is very little genetic variation in the species as a whole. The transfer of a few individuals between stocks and thus the maintenance of genetic homogeneity across larger geographical scales has very little practical consequences for fishery management; fish populations that are not reproductively isolated can still constitute appropriate management units, provided they respond independently to the effects of exploitation (Pawson & Jennings 1996). Thus, studies that detect environmental signals, such as parasites, otolith elemental composition, or fatty acid profiles, have the potential to be particularly valuable from a fishery management perspective (Cadrin et al. 2005).

Finally, some methods of stock identification rely on artificial tagging of fish. This usually involves a mark and recapture protocol, and as such is very costly and somewhat inefficient, but recent advances in electronic tagging and telemetry have dramatically improved the power of such techniques, which can offer valuable information on distribution, migration, and specific behaviour, especially of larger fish (Bain 2005).

Parasites as biological tags

The principle of the use of parasites as biological tags for the study of fish populations is that fish become infected with a parasite only when they come within the endemic area of that parasite (MacKenzie & Abaunza 2005). Parasite distributions are restricted to the geographical regions that are suitable for their transmission and life cycle completion.

Parasitological approaches to stock identification can provide excellent indicators of stock separation (Pawson & Jennings 1996). This method offers several advantages in comparison both with artificial tagging and genetic methods (MacKenzie & Abaunza 2005). The use of parasite tags is potentially applicable to all fish, including small and delicate species that are not always possible to tag effectively, and it also obviates the concern with possible abnormal behaviour of tagged fish. Moreover, it is a cheaper method, requiring a much smaller sampling effort, since each sampled specimen constitutes a valid observation, whereas with artificial tags an individual must be sampled, tagged, and recaptured in order to obtain a valid obervation (MacKenzie & Abaunza 2005). The use of parasites as biological tags is one of the non-genetic based methods that can be particularly useful to identify stocks that present low genetic differentiation but can nevertheless constitute separate populations with their own particular dynamics of reproduction, recruitment, and response to fishing pressure.

The limitations of this method are mostly related to the lack of information on the ecology and biology of many parasites species, which can limit their efficient use as biological tags (MacKenzie & Abaunza 2005). In particular, information about the life span of many parasite species is uncertain. Many parasite species also present difficulties regarding their correct identification. In fact, the taxonomic resolution in parasitological studies has been decreasing in recent years, due in part to a worldwide decline in parasite taxonomic expertise (Poulin & Leung 2010). Furthermore, some of the most useful parasites for biological tagging, such as anisakid larvae, can only be positively identified by the use of molecular biology techniques, which can be costly and time-consuming.

The successful use of parasites as biological tags for the identification of fish stocks is subject to the careful selection of parasites according to pre-defined criteria, of which the most important are the following (MacKenzie & Abaunza 1998, 2005, Lester & MacKenzie 2009):

- Infection levels (prevalence, intensity or abundance of infection, as defined by Bush et al. (1997)) should be different in the subject host in different parts of the study area;
- It should persist on the host for a long period of time, preferably more than a year;
- Parasites that are serious pathogens should be avoided, especially if they influence host behaviour.

The selection of appropriate parasite tags is contingent on the available information on the parasite fauna of the host in the study area; thus, if little information is available, it is necessary to begin with a preliminary study of the whole parasite fauna and only then proceed to the selection of adequate parasite tags (MacKenzie & Abaunza 2005). Another approach is to use whole parasite assemblages, which requires fewer individual fish to be sampled and observed, and is therefore more appropriate for large and valuable fish species (MacKenzie & Abaunza 2005). However, even when using parasite assemblages, temporary parasites should be discarded as they can be acquired and lost many times in the course of the host's life span, and only parasites that remain on the fish host for long periods of time should be used (Lester & MacKenzie 2009).

POPULATION STRUCTURE OF *Pagellus bogaraveo*

Lack of differentiation between Atlantic and Mediterranean populations

Studies on the population structure of *P. bogaraveo* have uncovered a lack of genetic differentiation between fish from the Atlantic and Mediterranean Sea, in stark contrast with other sparid species (Bargelloni et al. 2003, Patarnello et al. 2007). Bargelloni et al. (2003) surveyed population structure in several sparid species using the same mitochondrial fragment (D-loop) and the same set of allozyme markers. While some species evidenced a sharp Atlantic-Mediterranean separation, *P. bogaraveo* and *Pagrus pagrus* showed very little or no population structure and no sign of Atlantic-Mediterranean division (Bargelloni et al. 2003). *P. bogaraveo* exhibited a shallow mitochondrial DNA network, with a star-like shape, characterized by several low-frequency haplotypes stemming from the most common one (Bargelloni et al. 2003). In addition, mismatch analysis of sequences (frequency of pairwise differences between haplotypes) shows a skewed unimodal distribution consistent with a recent bottleneck or sudden population expansion (Patarnello et al. 2007). The putative population expansion was calculated to be no older than the late Pleistocene, a period characterized by strong climatic changes (Patarnello et al. 2007).

This lack of genetic differentiation could be due to high gene flow, as in pelagic and highly migratory species such as *Scomber scombrus*, but it seems more likely, in the case of the benthopelagic *P. bogaraveo*, to be the consequence of recent historical events (Bargelloni et al. 2003). It has been hypothesized that blackspot seabream populations in the Mediterranean might have been driven to extinction during the Pleistocene, and the present lack of genetic differentiation might be the result of recolonization of the Mediterranean from the Atlantic, although the opposite is also possible (Bargelloni et al. 2003; Patarnello et al. 2007), and there has not been enough time for the populations to diverge again, even if migration across the Gibraltar Strait is very limited, as seems to be the case for other sparids.

Palma & Andrade (2004) detected significant differences in morphometric measurements of four *P. bogaraveo* populations, from Azores and three Mediterranean localities; unexpectedly, though, the Italian sample, not the Azorean, was the most different, and no geographical gradient was detected.

Piñera et al. (2007) studied the population structure of *P. bogaraveo* in Spanish waters using microsatellite markers, comparing samples from the Mediterranean and Atlantic (Galicia and Cantabrian Sea). Results showed no differentiation between samples, and the authors concluded that the lack of differentiation among different localities within the same continental slope might be due to the absence of barriers to gene flow or, if such barriers exist, that they have not been strong enough to generate or preserve significant genetic differentiation between these areas (Piñera et al. 2007).

Population structure in the north-east Atlantic

In contrast with the apparent lack of genetic differentiation between Atlantic and Mediterranean *P. bogaraveo* populations, there appears to be some population structure in the north-east Atlantic, albeit low to moderate.

The study by Stockley et al. (2005) confirms the results of Bargelloni et al. (2003) regarding low genetic variability in mitochondrial DNA in this species. The cytochrome *b* gene presents a complete lack of variation among *P. bogaraveo* from several locations both in the north-east Atlantic and the Mediterranean (Stockley et al. 2005, Lemos et al. 2006), which is highly unusual and is comparable to species that are known to have undergone extreme population bottlenecks, suggesting that blackspot seabream effective population size must have been reduced to very low numbers sometime in the past (Stockley et al. 2005). However, recent effects of fishing, especially severe mortality amongst females, could also have resulted in low maternal population size and hence contributed to the low diversity in the mitochondrial genome (Stockley et al. 2005).

Stockley et al. (2005) also detected low genetic variability in another mitochondrial region, D-loop sequences, which nevertheless indicated significant, albeit low, genetic differentiation between populations at a regional level, specifically between the Azores and Peniche/Madeira. Microsatellite markers exhibited differentiation between the Azores and the continental slope, whereas there was no differentiation between island groups within the Azores archipelago (Stockley et al. 2005). These results suggest the existence of some population structure in the north-east Atlantic, and are consistent with studies indicating that demersal and benthopelagic species that inhabit continental slopes and seamounts tend to show limited dispersal between geographic areas on an oceanic, regional, and even more local scale (Rogers et al. 2005). In particular, the ocean basin between the Azores and the European continental shelf may limit dispersal of *P. bogaraveo* within the north-east Atlantic (Piñera et al. 2007).

In conclusion, studies seem to point to a very low genetic variability of *P. bogaraveo* across its geographical range and little or no differentiation between Atlantic and Mediterranean populations, consistent with a Pleistocene bottleneck and subsequent population expansion, and low but significant levels of population differentiation in the north-east Atlantic that point to the existence of two stocks separated by the ocean basin between the European continental shelf and the Azores.

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Fig. 1. Blackspot seabream, *Pagellus bogaraveo*.

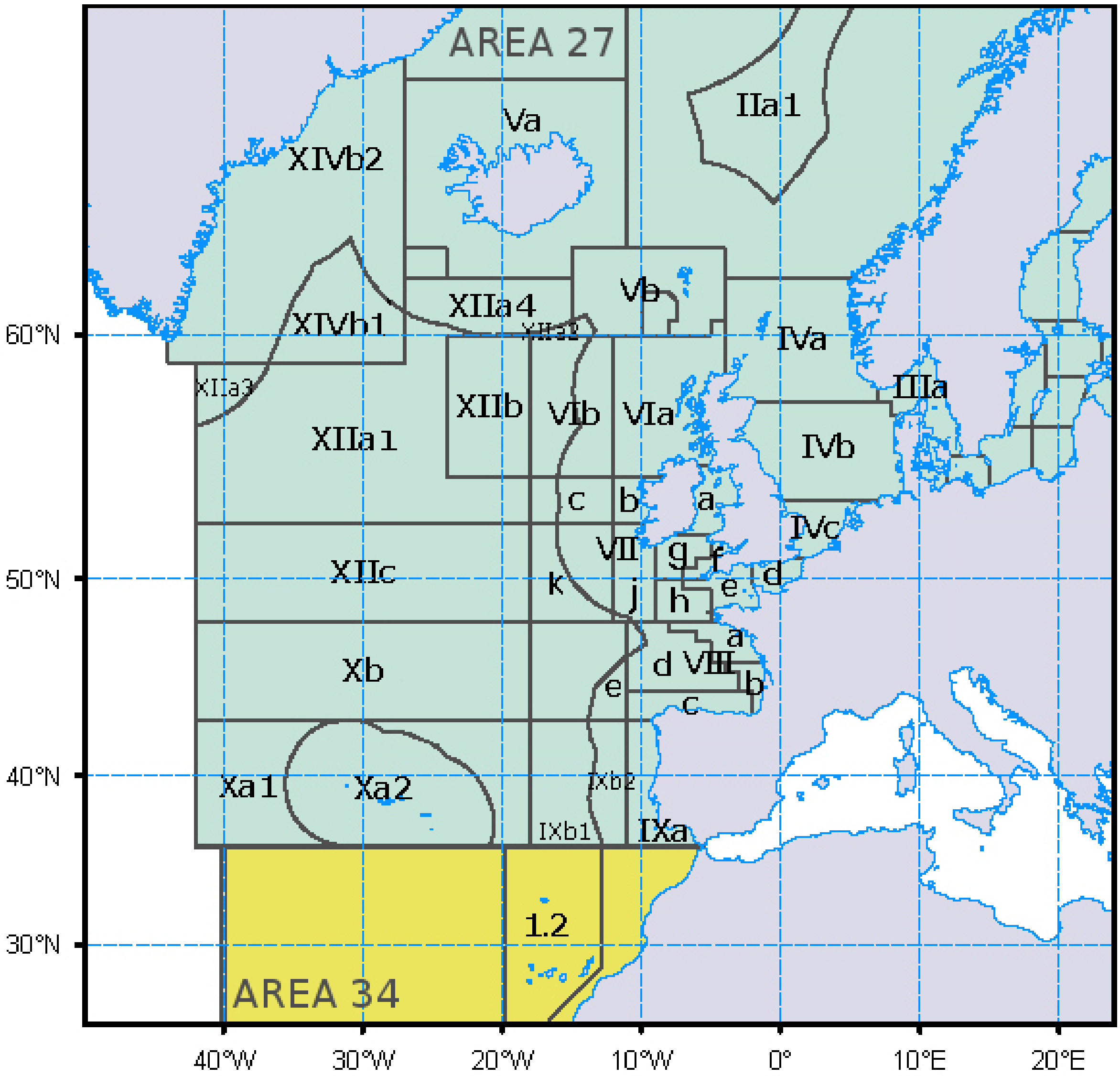

Fig. 2. Fishing areas of the north-east Atlantic: FAO Area 27 with ICES divisions and northern part of FAO Area 34 which includes Madeira.

MAIN AIMS OF THIS STUDY

- To describe the parasite fauna of *Pagellus bogaraveo*, which is poorly known;
- To assess the occurrence and parasite infection levels in fish from different localities within the Portuguese Exclusive Economic Zone (EEZ);
- To determine the occurrence of appropriate parasites to be used as biological tags for the identification of blackspot seabream stocks in the north-east Atlantic;
- To select and use adequate parasites as biological tags to identify stocks of *P. bogaraveo* in the north-east Atlantic.

CHAPTER 2

Ectoparasites

FISH ECTOPARASITES

Ectoparasites of marine fish

Fish ectoparasites generally have direct life cycles, involving only a single host. Several taxonomic groups include ectoparasites of marine fish, the most common being the monogeneans and crustaceans. The Monogenea as a group is almost entirely composed of fish ectoparasites, commonly living on the gills or skin of fish. It includes the Monopisthocotylea, which have a posterior attachment organ (opisthaptor) usually consisting of hooks or sometimes a sucker, and the Polyopisthocotylea, which attach by means of a complex clamp system, and in some cases also hooks (Rohde 1993).

There are several groups within the Crustacea that are fish ectoparasites, although some are only parasitic during particular stages of their life cycle. Copepods are among the most common ectoparasites of marine fish. Most copepods have direct life cycles with a free living larval stage which actively seeks the host. Branchiurans such as *Argulus* spp. are most commonly known as freshwater parasites, but some species also infect marine fish. Several isopods also parasitize fish; the Gnathiidae are parasitic during larval stages, feeding on fish blood, whereas adults are free-living and do not feed. Aegid isopods are likewise temporary fish parasites.

Temperature is one of the major factors responsible for the geographical distribution of marine organisms, including parasites (Rohde 1984). Ectoparasites living on the skin, gills, nostrils, and mouth of fish are especially vulnerable to sea water temperature, and therefore their prevalence and abundance in different regions is likely to be conditioned by this factor. In particular, higher temperatures are known to favour proliferation of many ectoparasite species, whereas low temperatures are often limiting (e.g. Jones 1998).

Impact of ectoparasites on aquaculture

Fish parasites can often have a severe impact on aquaculture. Ectoparasites, in particular, are especially prone to proliferate under farming conditions (Nowak 2007). On the one hand, the stress of confinement and crowding conditions can render fish more vulnerable to parasite infection by lowering their natural resistance (Conte 2004) and, on the other hand, normal behavioural measures of parasite avoidance, such as habitat selection and seeking cleaner fish, are unavailable to cultured fish (Barber 2007; Grutter 2003). Whereas farmed fish are relatively free from most endoparasites, as they have little opportunity to ingest the infective stages, which are usually trophically acquired, the infective stages of ectoparasites are often free in the water column, and can also be transmitted from one fish to another.

Even parasites that have little or no impact on wild fish can easily reach very significant numbers when conditions are appropriate for their development. Many ectoparasites feed on blood and, if present in large numbers, can cause anaemia and reduced growth rates. Gill parasites can cause damage to the gill and promote an excessive production of mucus on the part of the host and, when present in large numbers, may impair normal respiratory function, which can in extreme cases lead to the death of the host by hypoxia (Marino et al. 2004). In addition, ectoparasites can often cause lesions that are susceptible to subsequent bacterial or fungal infection.

The study of the natural parasite fauna of *P. bogaraveo*, especially of ectoparasites, is relevant not only to further our knowledge of the natural susceptibility of blackspot seabream to different parasites, but also from an aquaculture perspective, since wild fish tend to be attracted to floating structures at sea, and to aggregate around them, thus facilitating transmission of parasites between wild and farmed fish (Dempster et al. 2004).

ECTOPARASITES OF BLACKSPOT SEABREAM

Ectoparasite communities

In the first paper (Hermida M., Cruz C., Saraiva A. Ectoparasites of the blackspot seabream *Pagellus bogaravo* (Teleostei: Sparidae) from Portuguese waters of the north-east Atlantic, *Journal of the Marine Biological Association of the United Kingdom, in press*), the ectoparasite fauna of *P. bogaraveo* from Portuguese waters of the north-east Atlantic is described. Nine ectoparasite species were detected. The monopisthocotylean monogenean *Lamellodiscus virgula* (Fig. 3) occurred in all sampled localities, occasionally with very high intensity. Another monogenean, the polyopisthocotylean *Choricotyle chrysophryi* (Fig. 4), occurred only in mainland samples, with low infection levels. Very high infection levels of the copepod *Hatschekia pagellibogneravei* (Fig. 5), another gill parasite, were detected in Madeira, whereas infection levels were much lower in the Azores and it was absent from mainland waters. The only other copepod species detected was *Peniculus fistula*, of which only two specimens were found, attached to the caudal fin of the host. Several isopods were detected, all of which are temporary parasites of fish and are likely to show little or no host specificity. Praniza larvae of *Gnathia* sp. (Fig. 6) and the aegids *Aega deshaysiana*, *A. antillensis*, and *Rocinela danmoniensis* (Fig. 7), were all detected for the first time in this host, as was the branchiuran *Argulus* sp., of which a single specimen was detected.

Blackspot seabream from Madeira had significantly higher infection levels (both prevalence and intensity) of ectoparasites, crustaceans, and of the copepod *H. pagellibogneravei*. These differences are consistent with findings of increased abundance of ectoparasites of marine fish with decreasing latitude (Rohde & Heap 1998) and are related to differences in water temperature range. Mainland waters were characterized by higher prevalence of monogeneans, which might be related to higher pollution levels of continental waters when compared to open oceanic waters.

Microhabitat distribution of *Hatschekia pagellibogneravei* on the gills

During the study of the ectoparasite fauna of *P. bogaraveo*, a very large population of the copepod *Hatschekia pagellibogneravei* was detected in blackspot seabream from Madeiran waters. In the second paper (Hermida M., Cruz C., Saraiva A. (2012) Distribution of *Hatschekia pagellibogneravei* (Copepoda: Hatschekiidae) on the gills of *Pagellus bogaraveo* (Teleostei: Sparidae) from Madeira, Portugal, *Folia Parasitologica*, 59: 148-152), the microhabitat distribution of this copepod on the gills of blackspot seabream was analysed.

No significant differences were detected between the left and right gills, but significant differences were detected between gill arches. The uneven distribution of gill parasites is a well-known phenomenon for which several explanations have been advanced. In this study, the distribution pattern of *H. pagellibogneravei* on the four gill arches is more likely to be explained by differences in water flow within the gill habitat, although other hypotheses are discussed. Interestingly, a negative correlation between *H. pagellibogneravei* abundance and fish size was detected, which was unexpected, but might be related to ontogenetic habitat changes on the part of blackspot seabream, since older fish tend to prefer deeper waters, which are colder, low temperature being a limiting factor for many copepods.

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Fig. 3. (above)
Lamellodiscus virgula
(Monogenea: Diplectanidae).

Fig. 4 (left)
Choricotyle chrysophryi
(Monogenea: Diclidophoridae).

Fig. 5. (above) *Hatschekia pagellibogneravei* (Copepoda: Hatschekiidae). A, whole body; B, detail of posterodorsal protuberance on the cephalothorax.

Fig. 6. (left)
Gnathia sp. (Isopoda: Gnathiidae)

Fig. 7. (below)
Rocinela danmoniensis (Isopoda: Aegidae)

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Ectoparasites of the blackspot seabream *Pagellus bogaraveo* (Teleostei: Sparidae) from Portuguese waters of the north-east Atlantic

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The ectoparasite community of the blackspot seabream, Pagellus bogaraveo*, was studied in different locations in Portuguese waters of the north-east Atlantic Ocean. This is the first study to focus on the ectoparasites of this commercially important sparid fish. Nine ectoparasite species were detected.* Gnathia *sp.,* Aega deshaysiana, A. antillensis, Rocinela danmoniensis *and* Argulus *sp. are reported for the first time on this host. Significant differences were detected among the sampling locations, with monogeneans being more prevalent in mainland waters, and crustaceans being more prevalent in the Atlantic islands of Madeira and Azores. Fish from Madeira showed significantly higher infection levels of all ectoparasites, especially crustaceans, and particularly high prevalence of* Hatschekia pagellibogneravei*. The potential impact of the species detected on captive fish is also discussed, since the blackspot seabream is a promising new species for marine aquaculture.*

Keywords: *Pagellus bogaraveo*, blackspot seabream, Portugal, north-east Atlantic, ectoparasites

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INTRODUCTION

The blackpot seabream, *Pagellus bogaraveo* (Brünnich, 1768) is a commercially important benthopelagic sparid fish that occurs in the north-east Atlantic as well as the Mediterranean Sea. Its parasitic fauna is insufficiently known, and there has never been a comprehensive study of its ectoparasites. There are some studies of specific parasite groups that report ectoparasite species detected in *P. bogaraveo*. The monogeneans *Lamellodiscus virgula* Euzet & Oliver, 1967, *Encotyllabe pagelli* van Beneden & Hesse, 1863, *Choricotyle chrysophryi* (van Beneden & Hesse, 1863) and *Choricotyle pagelli* Llewelyn, 1941 have been reported from blackspot seabream from north-east Atlantic waters (Llewellyn, 1941, 1956; Oliver, 1973; Hansson, 1998). Several copepod species have also been reported on *P. bogaraveo*, but mostly from the Mediterranean (Raibaut *et al.*, 1998); in the north-east Atlantic only three copepods have been detected parasitizing this fish, namely *Caligus centrodonti* Baird, 1850, *Hatschekia pagellibogneravei* (Hesse, 1878), and *Peniculus fistula* von Nordmann, 1832 (Scott & Scott, 1913; Gooding, 1957; Jones, 1985). The cymothoid isopod *Ceratothoa collaris* Schiödte & Meinert, 1883 has also been detected on this fish (Bariche & Trilles, 2008).

This study is the first attempt to describe the ectoparasite community of *P. bogaraveo* in the wild, and also to evaluate whether these parasites might become problematic in captivity, since this fish has recently started to be produced in aquaculture (Basurco *et al.*, 2011), and seems to be an excellent alternative to other commonly cultured sparids, such as *Sparus aurata*, due to its higher market value (Peleteiro *et al.*, 2000). Skin and gill parasites often have serious impact in cultured fish because, on the one hand, captive fish often experience higher stress levels than wild fish, rendering them more vulnerable to the negative effects of parasitism and, on the other hand, they are limited in the behavioural ways in which they might normally avoid the parasites that are present in the environment, or minimize their impact by seeking cleaner fish (Grutter, 2003). Skin parasites often cause lesions that might lead to secondary infections, especially of bacterial aetiology. Gill parasites, especially when present in large numbers, can cause severe damage to the gill and impair normal respiratory function, which may lead to the death of the host by hypoxia (Marino *et al.*, 2004). In addition, many ectoparasites feed on blood, and high intensities of infection may lead to anaemia and poor growth rates.

Another important aim of this study was to assess the differences in the ectoparasite communities of *P. bogaraveo* from different locations in the Portuguese waters, especially between mainland Portugal and the Atlantic islands of Madeira and the Azores. The fact that different locations were analysed, and also that sampling was carried out throughout the year, may have helped to capture a wide range of parasites that might not have been detected had the study been carried out in a single location or during a specific

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time of the year, and might help to achieve a more comprehensive perspective of the ectoparasites of *P. bogaraveo* in Atlantic waters.

MATERIALS AND METHODS

A total of 348 specimens of *Pagellus bogaraveo* caught in the Portuguese Exclusive Economic Zone were acquired from commercial catches between the years 2009 and 2011, from four mainland locations (42 from Matosinhos, 92 from Figueira da Foz, 42 from Peniche and 30 from Sagres) and the Atlantic islands of Madeira (56) and Azores (86) (Figure 1). Sampling was carried out during two seasonal periods (autumn/winter and spring/summer) in all locations except Sagres, where only spring/summer samples could be obtained. Fish were transported in ice to the laboratory and measured before being frozen in individual plastic bags. Fish mean length is presented in Table 1. After defrosting, each fish was analysed for the presence of ectoparasites. The tegument, mouth, nostrils and gill chambers were thoroughly observed. The gills were removed and placed in Petri dishes and each gill arch was separately observed under a stereomicroscope. All parasites were collected and preserved in 70% ethanol. Monogeneans were cleared and mounted in glycerol, observed in an optical microscope and identified according to the descriptions of Llewellyn (1941), Dawes (1947) and Oliver (1973). Identification of copepods followed Kabata (1979), Alexander (1983) and Boxshall (1986). Isopods were identified according to Sars (1899), Richardson (1905), Norman & Scott (1906) and Bruce (2004).

Prevalence and intensity of infection were determined according to Bush *et al.* (1997). Statistical analyses were carried out using IBM SPSS statistics software. Whenever possible, prevalence and intensity of parasite infection were compared among all 6 locations, and also between three regions (mainland, Madeira and Azores), using a Chi-square test for prevalence, and the non-parametric Kruskal–Wallis test for intensities, followed by multiple comparisons whenever significant differences were detected. For all tests, statistical significance was accepted when $P < 0.05$.

RESULTS

A total of nine ectoparasite species were detected in the 348 specimens of *Pagellus bogaraveo* examined: the monogeneans *Lamellodiscus virgula* and *Choricotyle chrysophryi*; the copepods *Hatschekia pagellibogneravei* and *Peniculus fistula*; the isopods *Gnathia* sp. Leach, 1814 (praniza larvae), *Aega deshaysiana* (Milne Edwards, 1840), *Aega antillensis* Schiöedte & Meinert, 1879 and *Rocinela danmoniensis* Leach, 1818; and the branchiuran *Argulus* sp. Müller, 1785. Prevalence and intensity of infection for each species is presented in Tables 1 and 2, respectively.

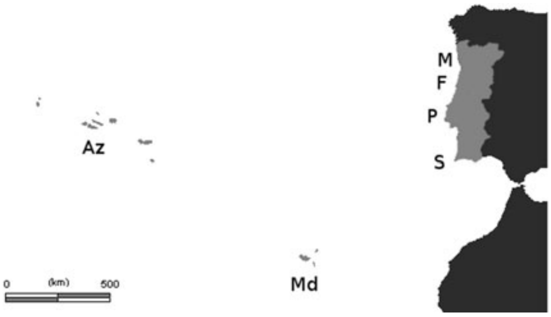

Fig. 1. Sampling locations on the Portuguese mainland coast: (M) Matosinhos; (F) Figueira da Foz; (P) Peniche; (S) Sagres; and Islands: (Md) Madeira; (Az) Azores.

Only 113 fish (32%) of all the specimens observed were infected with at least one ectoparasite species. Of these, the majority (81%) harboured only one species, whereas 15% had two parasite species and 4% three. No more than three species were detected per host.

Monogeneans were located exclusively on the gills. Prevalence of monogeneans was significantly different in the three regions, being higher in mainland samples. *Lamellodiscus virgula* was the only parasite species that occurred in all sampling locations, with a maximum prevalence in Sagres (30.0%). Intensity of infection was highly variable, but no significant differences were detected between locations. A maximum of 772 individuals were observed on a single fish. The polyopisthocotylean *C. chrysophryi* was detected in all mainland locations, but not in the islands. Prevalence was again highest in Sagres, but reached only 6.7%. Intensity of infection was always very low; no more than two specimens were ever detected in a single host.

The copepod *H. pagellibogneravei*, another gill parasite, presented the highest prevalence detected in this study: 96.4% in Madeira waters. This species also occurred in the Azores, albeit with a much lower prevalence (5.8%), but was absent from mainland waters. Intensity of infection was also significantly higher in Madeira. The copepod *P. fistula* was found attached to the caudal fin of only two fish, producing a dark colour lesion in the attachment site.

Praniza larvae of *Gnathia* sp. were detected in the mouth, oesophagus, gills, gill chamber, and on the tegument and fins of several fish from the islands and from one mainland location. Prevalence of this isopod was significantly higher in the islands. Mean intensity was not particularly high, but occasionally a single fish hosted more than 100 *Gnathia* sp. Three aegid isopods were detected for the first time in *P. bogaraveo*: *A. deshaysiana*, *A. antillensis* and *R. danmoniensis*. These isopods were usually detected on the tegument, most often underneath the pectoral fins, with the exception of *A. antillensis*, which was located on the gills, and infection levels were always low. A single specimen of *Argulus* sp. was also detected under a pectoral fin on a fish from Azorean waters.

Globally no significant differences were detected between autumn/winter and spring/summer except in the prevalence of *Gnathia* sp. (autumn/winter = 17.6% and spring/summer = 6.3%) and *H. pagellibogneravei* (autumn/winter = 25.2% and spring/summer = 10.1%), and intensity of *Gnathia* sp (mean intensity autumn/winter = 2.2 and spring/summer = 20.2).

Although nine ectoparasite species were detected, species richness never exceeded 5 at any sampling location. Fish from both islands had an ectoparasite community composed of 4 crustaceans and one monogenean species; while in mainland waters the ectoparasite community was composed of 3 crustaceans and 2 monogeneans. Overall, monogenean prevalence was higher in the mainland, whereas prevalence of crustaceans was higher in the islands. Within the three studied regions, prevalence and intensity of crustaceans and ectoparasites were significantly higher in Madeira.

Table 1. Prevalence (%) of parasite infection in *Pagellus bogaraveo* from locations on mainland Portugal (Matosinhos, Figueira da Foz, Peniche and Sagres) and Islands (Madeira and Azores). Number (N) and length (L) (mean ± standard deviation, in cm) of fish examined. Significant differences detected among locations and among regions (mainland, Madeira and Azores) by Chi-square test (similar letters indicate no significant differences).

Parasite species	**Matosinhos N = 42 L = 32.0 ± 1.7**	**Figueira da Foz N = 92 L = 33.0 ± 1.8**	**Peniche N = 42 L = 37.4 ± 3.1**	**Sagres N = 30 L = 30.0 ± 0.7**	**Madeira N = 56 L = 31.0 ± 2.6**	**Azores N = 86 L = 34.9 ± 4.7**	**Total mainland N = 206 L = 33.2 ± 3.1**	**Overall N = 348 L = 33.3 ± 3.7**	**Significant differences**
Lamellodiscus virgula	9.5	2.2	11.9	30.0	1.8	1.2	9.7	6.3	
					ab	a	b		3 reg: $P = 0.007$
Choricotyle chrysophryi	4.8	1.1	2.4	6.7	0.0	0.0	2.9	1.7	
TOTAL MONOGENEA	14.3	3.3	14.3	36.7	1.8	1.2	12.6	8.0	
					ab	a	b		3 reg: $P = 0.001$
Hatschekia pagellibogneravei	0.0	0.0	0.0	0.0	96.4	5.8	0.0	17.0	
	a	a	a	a	b	a			6 loc: $P = 0.000$
					c	b	a		3 reg: $P = 0.000$
Peniculus fistula	0.0	1.1	0.0	3.3	0.0	0.0	1.0	0.6	
Gnathia sp.	0.0	10.9	0.0	0.0	21.4	20.9	4.9	11.5	
					b	b	a		3 reg: $P = 0.000$
Aega deshaysiana	0.0	0.0	0.0	0.0	7.1	0.0	0.0	1.1	
Aega antillensis	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.3	
Rocinela danmoniensis	0.0	1.1	2.4	0.0	5.4	0.0	1.0	1.4	
Argulus sp.	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.3	
TOTAL CRUSTACEA	0.0	12.0	2.4	3.3	96.4	24.4	6.3	25.3	
	a	ab	a	ab	c	b			6 loc: $P = 0.000$
					c	b	a		3 reg: $P = 0.000$
TOTAL ECTOPARASITES	14.3	14.1	16.7	36.7	96.4	25.6	18.0	32.5	
	a	a	a	a	b	a			6 loc: $P = 0.000$
					b	a	a		3 reg: $P = 0.000$

P, probability level.

Table 2. Intensity (median, mean ± standard deviation, (range)) of parasite infection in *Pagellus bogaraveo* from locations in mainland Portugal (Matosinhos, Figueira da Foz, Peniche and Sagres) and Islands (Madeira and Azores). Significant differences detected among locations and among regions (mainland, Madeira and Azores) by the Kruskal–Wallis test (similar letters indicate no significant differences).

Parasite species	Matosinhos	Figueira da Foz	Peniche	Sagres	Madeira	Azores	Total mainland	Overall	Significant differences
Lamellodiscus virgula	13.5 20.0 ± 24.0 (1–52)	1.5 1.5 ± 0.7 (1–2)	58.0 182.0 ± 331.3 (1–772)	11.0 17.3 ± 16.4 (1–47)	90.0 90.0 (90)	45.0 45.0 (45)	10.5 57.4 ± 169.7 (1–772)	12.5 58.4 ± 161.6 (1–772)	
Choricotyle chrysophryi	1 1.0 ± 0.0 (1)	1 1.0 (1)	1 1.0 (1)	1.5 1.5 ± 0.7 (1–2)	–	–	1.0 1.2 ± 0.4 (1–2)	1.0 1.2 ± 0.4 (1–2)	
TOTAL MONOGENEA	1.5 13.7 ± 21.0 (1–52)	1.0 1.3 ± 0.6 (1–2)	32.0 151.8 ± 305.4 (1–772)	10.0 14.5 ± 16.0 (1–47)	90.0 90.0 (90)	45.0 45.0 (45)	3.5 44.5 ± 149.9 (1–772)	5.0 46.1 ± 144.5 (1–772)	
Hatschekia pagellibogneravei	–	–	–	–	17.5 35.3 ± 63.2 (1–363)	4.0 4.2 ± 2.4 (1–7)	–	15.0 32.6 ± 61.1 (1–363)	
	–	–	–	–	b	a			2 loc: P=0.020
					b	a	–		2 reg: P=0.020
Peniculus fistula	–	1.0 1.0 (1)	–	1.0 1.0 (1)	–	–	1.0 1.0 ± 0.0 (1)	1.0 1.0 ± 0.0 (1)	
Gnathia sp.	–	3.0 7.0 ± 10.2 (1–31)	–	–	1.0 2.3 ± 2.9 (1–11)	2.0 11.5 ± 30.9 (1–133)	3.0 7.0 ± 10.2 (1–31)	2.0 7.6 ± 21.4 (1–133)	
Aega deshaysiana	–	–	–	–	1.0 1.5 ± 1.0 (1–3)	–	–	1.0 1.5 ± 1.0 (1–3)	
Aega antillensis	–	–	–	–	–	1.0 1.0 (1)	–	1.0 1.0 (1)	
Rocinela danmoniensis	–	1.0 1.0 (1)	1.0 1.0 (1)	–	1.0 1.0 ± 0.0 (1)	–	1.0 1.0 ± 0.0 (1)	1.0 1.0 ± 0.0 (1)	
Argulus sp.	–	–	–	–	–	1.0 1.0 (1)	–	1.0 1.0 (1)	
TOTAL CRUSTACEA	–	2.0 6.5 ± 9.8 (1–31)	1.0 1.0 (1)	1.0 1.0 (1)	17.5 35.9 ± 63.2 (1–363)	4.0 11.0 ± 28.5 (1–133)	2.0 5.7 ± 9.2 (1–31)	8.0 25.5 ± 53.0 (1–363)	
	–	ab	abc	abc	C	b			6 loc: P=0.000
					b	a	a		3 reg: P=0.000
TOTAL ECTOPARASITES	1.5 13.7 ± 21.0 (1–52)	2.0 5.8 ± 9.1 (1–31)	6.0 130.3 ± 284.6 (1–772)	10.0 14.5 ± 16.0 (1–47)	19.0 37.6 ± 63.9 (1–363)	4.0 12.5 ± 28.7 (1–133)	3.0 33.2 ± 126.2 (1–772)	8.0 31.3 ± 85.4 (1–772)	
	ab	a	ab	ab	b	a			6 loc: P=0.001
					b	a	a		3 reg: P=0.000

P, probability level.

DISCUSSION

Parasite species detected

Until recently, two morphologically very similar species of diplectanid monogeneans were thought to occur in *Pagellus bogaraveo*, namely *Lamellodiscus obeliae* and *Lamellodiscus virgula*; however, analysis of their ribosomal DNA sequences has revealed differences of less than 1%, and they are therefore now considered a single species, *L. virgula* (Desdevises *et al.*, 2000). This monogenean occurred in fish from all locations studied, but it was more common in the mainland. Prevalence values were usually lower than those observed by Oliver (1973) on the Atlantic coast of France (30%) and by Kaouachi *et al.* (2010) in the Mediterranean (12–33%), on the same host. Although less pathogenic than other diplectanid species such as *Diplectanum* spp., *Lamellodiscus* spp. have been shown to cause damage to the gills, especially epithelial hyperplasia and microhaemorrhages (Sánchez-García *et al.*, 2011).

Choricotyle chrysophryi is a polyopisthocotylean that has previously been recorded in *P. bogaraveo* from both the north-east Atlantic (Llewellyn, 1941, 1956) and the Mediterranean (Kaouachi *et al.*, 2010). It is a relatively large monogenean, whose attachment organs function as suckers, allowing the parasite to move freely on the surface of the gills (Llewellyn, 1956). Although polyopisthocotylean parasites of fish feed on blood, they generally tend to be harmless to their hosts in wild fish (Kearn, 2005). That is probably the case for this species, since intensity of infection seems to be usually very low. Llewellyn (1941) detected no more than two parasites per host, as was also observed in the present study, whereas Kaouachi *et al.* (2010) found no more than one. Nonetheless, it is likely that higher parasite loads could occur in captive conditions, and large numbers of blood feeding parasites could potentially cause anaemia.

Hatschekia pagellibogneravei is a relatively common copepod that parasitizes several species of sparid fish, including *P. bogaraveo* (Raibaut *et al.*, 1998; Boualleg *et al.*, 2010b). It has often been reported from the Mediterranean (Raibaut *et al.*, 1998; Ternengo *et al.*, 2009; Boualleg *et al.*, 2010a, 2010b), and occasionally also from the north-east Atlantic (Jones, 1985). In this study it was only detected in the Atlantic islands, with a significantly higher prevalence in Madeira. Boualleg *et al.* (2010a) found a relatively high prevalence of this parasite on *Diplodus annularis* (L., 1758) from the Mediterranean (60%) but, to the best of our knowledge, a prevalence reaching almost 100% had never been detected on any host. Intensity of infection was also higher than the highest mean intensity previously reported, 11.4 on *Diplodus cervinus* (Lowe, 1838) (Boualleg *et al.*, 2010b). A mean abundance of 6.8 is the only parasitological index provided for *H. pagellibogneravei* on *P. bogaraveo* in the Mediterranean (Boualleg *et al.*, 2010b), so here we also determined this parameter and again, mean abundance of this species in Madeira was much higher (34.0 ± 62.4).

Gnathiid isopods are obligatory fish parasites during three juvenile stages of praniza larvae, feeding on fish blood, whereas the adult stages are free-living and do not feed (Hadfield *et al.*, 2009). Most studies of wild fish populations found no significant pathology associated with *Gnathia* sp. infection (Heupel & Bennett, 1999; Gene, 2007); however they may transmit blood parasites such as *Haemogregarina bigemina* Laveran & Mesnil, 1901 (see Davies *et al.*, 2004). Many wild fish seek cleaner fish very frequently (up to more than 100 times per day), which may help to minimize the impact of gnathiids (Grutter, 2003). However, in captive fish, these isopods can cause not only anaemia (Jones & Grutter, 2005), but also severe mechanical damage to the skin and especially the gills, which may in extreme cases lead to death by hypoxia (Marino *et al.*, 2004). Although the intensity detected in some fish in the present study was occasionally high (reaching 133 parasites in one host), mean intensity was generally moderate, and no lesions associated with this parasite were observed. It should however be noted that the stress imposed on fish by capture, as well as handling and transport, may influence the prevalence and intensity of infection by these parasites, as they can easily detach from the host. Praniza larvae of *Gnathia* sp. had never been detected in *P. bogaraveo*, but they have often been reported in various fish species captured in Portuguese waters (e.g. Davies *et al.*, 1994; Marques *et al.*, 2006; Sequeira *et al.*, 2010). Not only do gnathiids have low host specificity, but they also seem to show no particular preference for any site of attachment (Hadfield *et al.*, 2009). In this study they were detected in different locations on the skin and fins, as well as in the mouth, gill chamber, and on the gills, which coincides with findings by other authors (e.g. Grutter, 2003).

Aegidae are temporary parasites of fish (Bunkley-Williams & Williams, 1998). Some authors refer to these temporary associations as ‘predation’ (e.g. Novotny & Mahnken, 1971; Bruce, 2004); however, aegids seem to be associated with fish for longer periods of time than strictly necessary for feeding (Bunkley-Williams & Williams, 1998). Aegids can have a negative impact on fish by causing large wounds, stunting growth, and occasionally even killing young fish (Bunkley-Williams & Williams, 1998). The temporary character of the association and the ease with which they can detach from the host (Rokicki, 1985) can help to explain the low number of Aegidae detected. In fact, for most aegid species, very few hosts have been reported, yet it seems likely that they exhibit low specificity. Bruce (2004) mentions two sharks as hosts of *A. deshaysiana*, and Ramdane & Trilles (2008) have recently detected it on *Sardina pilchardus* (Walbaum, 1792). The only host reported for *A. antillensis* was a serranid fish (Bruce, 2004). For *R. danmoniensis* Sars (1899) mentions that it is generally found ‘clinging to the skin of fishes of various kinds’. *Pagellus bogaraveo* is reported here for the first time as a suitable host for these three aegid species. Both *A. deshaysiana* and *R. danmoniensis* have been detected in the north-east Atlantic (Sars, 1899; Bruce, 2004), including in Madeiran waters, in the case of *A. deshaysiana*. On the other hand, the Portuguese mainland and Madeira extend the range of *R. danmoniensis* further south than was previously known. In the case of *A. antillensis*, the only previous records were from the north-west Atlantic, especially the Caribbean Sea and Gulf of Mexico (Bruce, 2004). However, the same author notes that the species is probably more widespread, and the present work extends its range of occurrence to the Azores, the westernmost archipelago of the north-east Atlantic.

Only two specimens of *P. fistula* were detected, both firmly attached to the caudal fin of their hosts. This was also the site of attachment reported by Gooding (1957). Only the post-metamorphose female is parasitic on fish, and exhibits a low host specificity (Boxshall, 1986). Attachment of this parasite involves a deep penetration of the tegument, and it seems likely that the resulting lesions might have a negative impact on fish health, particularly with higher intensities of infection.

Argulus sp. is a worldwide occurring branchiuran, commonly called fish lice. Several species are known to be highly pathogenic to cultured fish and, although there have been as yet no reports of *Argulus* sp. infections in marine aquaculture facilities, several marine species occur in the north-east Atlantic, and they could potentially have a severe negative impact on cultured fish (Schram *et al.*, 2005). There is currently some concern that *Argulus* sp. might start exhibiting higher growth rates in temperate regions due to climate warming (Hakalahti *et al.*, 2006). Here the blackspot seabream is reported for the first time as a suitable host for this crustacean.

Ectoparasite community

Although nine species of ectoparasites were detected in this study, species richness in any one location never exceeded 5 species. This is comparable to what has been reported for other sparid fish, such as *Boops boops* (L., 1758), *Dentex dentex* (L., 1758), *Diplodus vulgaris* (Geoffrow Saint-Hilaire, 1817) (all 6 ectoparasite species), and *Pagellus erythrinus* (L., 1758) (7 species) (González *et al.*, 2004; Pérez-del-Olmo *et al.*, 2007; Ternengo *et al.*, 2009). All of these species had only one or two monogeneans, as was also the case in the present study. Species richness of monogenean parasites of the genus *Lamellodiscus* is highly variable within the Sparidae, but in the larger species, such as *Sparus aurata* L., 1758, *Pagrus pagrus* (L., 1758) and *P. bogaraveo*, only one species usually occurs (Desdevises, 2006).

Copepod species richness is also highly variable, even within the genus *Pagellus*, with *Pagellus acarne* (Risso, 1827) being host to only two species and *P. erythrinus* to 13. *Pagellus bogaraveo* is somewhere in the middle, as seven copepod species have been reported from this fish (Raibaut *et al.*, 1998). In this study only two copepod species were detected, which is unexpectedly low, but one must bear in mind that most records of copepod parasites of *P. bogaraveo* that were not detected in the present study are from the Mediterranean Sea. However, these species (namely, *Alella pagelli* (Krøyer, 1863) and several *Caligus* spp.) are not unknown to occur in the north-east Atlantic (Kabata, 1992). No parasitological indices were found in the literature to assess whether these species are rare in the blackspot seabream, or whether they are more common in the Mediterranean than in the Atlantic.

In this study, monogeneans were more prevalent in Portuguese mainland waters. Several studies have shown that both monopisthocotylean and polyopisthocotylean monogeneans exhibit higher infection levels in polluted areas (see Pérez-del-Olmo *et al.*, 2007, and references therein). Since continental waters generally tend to be more polluted when compared to open oceanic waters, this seems a likely explanation for the higher prevalence of monogeneans in fish from Portuguese mainland waters.

Higher prevalence and intensity of ectoparasites, and crustaceans (i.e. all ectoparasites except monogeneans) in Madeira is a result that agrees with previous findings of increased abundance of ectoparasites of marine teleosts with decreased latitude (Rohde & Heap, 1998), and is related to differences in water temperature range. Rohde *et al.* (1995) found significant positive correlations between means of water temperature ranges (mean temperature during the coldest month to mean temperature during the warmest month) and intensity and abundance of ectoparasites, and abundance of copepods, specifically in benthopelagic fish. In this study, prevalence and intensity of ectoparasites, of crustaceans, and of the copepod *H. pagellibogneravei* were all significantly higher in Madeira, the southernmost location studied and the only one that is in a subtropical biogeographical region. In this area, the mean of the temperature range (data from the years 1981 to 2000) was 20.8°C, whereas in mainland Portugal it varied between 15.1°C in the northernmost location and 17.8°C on the southern coast, and in Angra do Heroísmo, Azores, it was 18°C (Carvalho & Soares, 2001).

In conclusion, nine ectoparasite species were detected on blackspot seabream from Portuguese waters, including two monogeneans and seven crustaceans, which is comparable to the ectoparasite communities of other sparid fish. *Gnathia* sp., *Aega deshaysiana*, *A. antillensis*, *Rocinela danmoniensis* and *Argulus* sp. were detected on this host for the first time. The ectoparasite community of *P. bogaraveo* varied significantly among locations, with the mainland samples showing higher prevalence of monogeneans. Madeira had significantly higher infection levels of ectoparasites, crustaceans, and the copepod *Hatschekia pagellibogneravei*, which might be due to biogeographical differences, as abundance of ectoparasites and copepods tends to increase in lower latitudes, especially in the case of benthopelagic fish.

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Distribution of *Hatschekia pagellibogneravei* (Copepoda: Hatschekiidae) on the gills of *Pagellus bogaraveo* (Teleostei: Sparidae) from Madeira, Portugal

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Abstract: A population of the gill parasite *Hatschekia pagellibogneravei* (Hesse, 1878) was studied on one of its sparid fish hosts, the blackspot seabream, *Pagellus bogaraveo* (Brünnich), off the coast of Madeira Island, Portugal, northeast Atlantic. Very high infection levels of this copepod were detected, with no significant seasonal differences. Abundance was negatively correlated with fish size. There were significant differences in the distribution of this copepod among the gill arches of the host, which seem to be best explained by differences in water flow within the gill habitat.

Keywords: copepoda, ectoparasites, marine fish, microhabitat, northeast Atlantic

The Hatschekiidae are a copepod family that comprises over 80 species, most of them tropical and subtropical (Kabata 1979). Despite the fact that these small copepods do not typically produce many eggs, they are remarkably successful, infecting numerous fish species, and sometimes aggregating in large numbers on their hosts (Jones 1998). They are characterized by a loss of definite segmentation in the region between the cephalothorax and the genital complex, which usually has a cylindrical shape. The males are much smaller than the females and they are unknown in numerous species (Kabata 1979, Jones 1985). *Hatschekia pagellibogneravei* (Hesse, 1878) (syn. *H. pagellibogueravei*) is a small and slender copepod that inhabits the gills of several fish of the family Sparidae (Raibaut et al. 1998, Boualleg et al. 2010a, 2011). It is easily distinguished from other members of the genus by its narrow, elongated form, and its characteristic postero-dorsal conical protuberance on the cephalothorax (Scott 1909, Scott and Scott 1913, Kabata 1979, Jones 1985). The blackspot seabream, *Pagellus bogaraveo* (Brünnich, 1768) (syns. *Sparus centrodontus*, *Pagellus centrodontus*), has been reported as a host for this parasite both in the Atlantic Ocean (Scott 1909, Scott and Scott 1913) and in the Mediterranean Sea (Boualleg et al. 2010a).

The uneven distribution of gill parasites among the gill arches of the fish host is a well known phenomenon that can be related to both endogenous and environmental factors (Price 1980). A few studies have considered the distribution of *Hatschekia* spp. on the gills of their hosts. Martens and Moens (1995) and Geets et al. (1997) have studied the distribution of *Hatschekia* sp. on the gills of *Siganus sutor* (Valenciennes) off the Kenyan coast, and Lo and Morand (2001) analysed the distribution of *Hatschekia* sp. on the gills of a coral reef fish, *Cephalopholis argus* Schneider, from French Polynesia. Scott-Holland et al. (2006) have studied the distribution of the asymmetrical *Hatschekia plectropomi* Ho et Dojiri, 1978 on the gills of the coral trout, *Plectropomus leopardus* (Lacépède) from Australia. There is also a study on the distribution of *Hatschekia hemigymni* Kabata, 1991 on the gills of *Hemigymnus melapterus* (Bloch) from Australia (Muñoz and Cribb 2005). However, no studies have been carried out concerning *H. pagellibogneravei* on any of its hosts. During a survey conducted on the parasite communities of *Pagellus bogaraveo* from Portuguese waters, a very abundant population of the copepod *H. pagellibogneravei* was detected on fish collected in the region of Madeira (Hermida et al. 2012). Therefore, a study on the microhabitat distribution of this copepod on the gills of its host was performed. The influence of factors such as host size and seasonality was also evaluated.

MATERIALS AND METHODS

A total of 56 specimens of *Pagellus bogaraveo* captured off the coast of Madeira Island, Portugal, northeast Atlantic (36 in autumn and 20 in spring) were acquired from commercial

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catches. Fish were weighed and measured, and subsequently frozen in individual plastic bags. After defrosting, the gills were carefully removed and the left and right gill arches placed in separate Petri dishes. Each gill arch was individually observed under a stereomicroscope. All *Hatschekia pagellibogneravei* specimens were collected, counted and preserved in 70% ethanol (v/v). Whenever some copepods remained on the Petri dish after all the gill arches had been removed, they were included in the total amount of parasites on that gill, but no attempt was made to assign them to any particular gill arch. Other parasites detected were also collected, counted, preserved in 70% ethanol (v/v), and identified, but no statistical analysis was performed due to their low numbers.

Statistical analyses were carried out using IBM SPSS statistics software. Fish size (weight and fork length), intensity and abundance of infection were compared between seasons (autumn versus spring) using the non-parametric Mann-Whitney test. Fork length was preferred to total length for statistical analyses because damage to the caudal fin prevented accurate measurement of total length in some cases. The correlation between fish size (weight and fork length) and parasite abundance was analysed by Spearman rank correlation. The parasite abundance in the right and left sides was compared for the entire gill (RG/LG) and for each gill arch (R1a/L1a; R2a/L2a; R3a/L3a; R4a/L4a) using the non-parametric Wilcoxon signed ranks test. Parasite abundance was compared among the 4 arches for the left gill (L1a/L2a/L3a/L4a), right gill (R1a/R2a/R3a/R4a), and for both gills (1A/2A/3A/4A), using the non-parametric Friedman's analysis of variance by ranks followed by multiple comparisons. For all tests, statistical significance was accepted when $p < 0.05$.

RESULTS

Of the 56 blackspot seabream observed, 54 were infected by the copepod *Hatschekia pagellibogneravei*, yielding an overall prevalence of 96%. Mean intensity and abundance were also high (35.3 and 34.0, respectively). No male copepods were detected. The maximum intensity observed was 363 copepods on the gills of a single fish. Host parameters and infection levels are presented in Table 1. There were no significant differences between seasons for either fish weight (Mann-Whitney non-parametric test; $p = 0.078$), fork length ($p = 0.245$), or *H. pagellibogneravei* infection levels, namely, intensity ($p = 0.332$) and abundance ($p = 0.436$).

Table 1. Host (*Pagellus bogaraveo*) size and infection levels by *Hatschekia pagellibogneravei* (prevalence, intensity and abundance). No significant differences were observed between seasons of any of the parameters analysed (non-parametric Mann-Whitney test).

N (fish)	Total 56	Autumn 36	Spring 20
Host weight (g):			
Mean ± SD	466 ± 140	426 ± 75	537 ± 195
Median	436	434	436
(Range)	(310–983)	(310–610)	(347–983)
Host fork length (cm):			
Mean ± SD	27.8 ± 2.2	27.4 ± 1.4	28.6 ± 3.0
Median	27.3	27.4	27.3
(Range)	(24.3–35.9)	(24.3–30.1)	(25.3–35.9)
Prevalence (%)	96.4	97.2	95.0
Intensity:			
Mean ± SD	35.3 ± 63.2	19.2 ± 15.1	64.8 ± 99.6
Median	17.5	16.0	19.0
(Range)	(1–363)	(1–54)	(1–363)
Abundance:			
Mean ± SD	34.0 ± 62.4	18.7 ± 15.2	61.6 ± 98.0
Median	16.0	15.0	17.5
(Range)	(0–363)	(0–54)	(0–363)

Abundance of *H. pagellibogneravei* was found to be significantly negatively correlated with fish size ($r = -0.279$, $p = 0.037$ for weight and $r = -0.281$, $p = 0.015$ for fork length). These correlations were also negative in spring ($r = -0.518$, $p = 0.019$ for weight and $r = -0.560$, $p = 0.010$ for fork length) and, although not statistically significant, in autumn ($r = -0.190$, $p = 0.266$ for weight and $r = -0.197$, $p = 0.249$ for fork length).

Abundance of *H. pagellibogneravei* on each particular arch and the significant differences detected are presented in Table 2. No significant differences in *H. pagellibogneravei* abundance were detected between the left and right gills either when analysed globally, or between corresponding gill arches. However, abundance of *H. pagellibogneravei* varied significantly between different arches. Arch number 2 was the most parasitised, followed by arch 3, then 1, and lastly 4. This pattern was observed on the left and right gills considered separately and also when both were combined. Gill arch number 4 was always significantly different from the second and third arches, and

Table 2. Distribution of *Hatschekia pagellibogneravei* on the gills of *Pagellus bogaraveo*. Statistically significant differences detected between gill arches on the left gill, right gill, and both gills combined by non-parametric Friedman's analysis of variance by ranks (X^2, p – probability level) followed by multiple comparisons (similar letters indicate no significant differences).

	Left gill						Right gill						Both gills					
Abundance	1L	2L	3L	4L	Unk.* L	Total L	1R	2R	3R	4R	Unk.* R	Total R	1L+R	2L+R	3L+R	4L+R	Unk.* L+R	Total L+R
Mean	3.9	5.0	3.9	1.9	1.9	16.6	3.5	5.0	4.4	2.2	2.3	17.4	7.3	10.1	8.3	4.1	4.2	34.0
SD	6.5	10.5	8.3	4.3	2.5	29.7	8.4	8.7	9.6	4.9	3.5	33.0	14.5	19.0	17.5	8.9	5.0	62.4
Median	1.0	1.5	1.0	0.0	1.0	7.0	1.0	2.0	1.5	1.0	1.0	8.5	2.0	4.5	3.0	1.0	2.0	16.0
Range	0–35	0–62	0–45	0–21	0–10	0–163	0–46	0–51	0–58	0–31	0–16	0–200	0–79	0–113	0–97	0–51	0–23	0–363
Significant differences	b	b	b	a			ab	b	b	a			b	b	b	a		
	$X^2 = 33.052$; $p = 0.000$						$X^2 = 27.437$; $p = 0.000$						$X^2 = 31.171$; $p = 0.000$					

* Parasites that were found loose and could not be assigned to a specific gill arch.

was also significantly different from the first both on the left gill and when data on both gills were combined.

The isopod *Gnathia* sp. was also detected on the gills of seven fish (prevalence of 13%) and the diplectanid monogenean *Lamellodiscus virgula* Euzet et Oliver, 1967 on one fish (prevalence of 2%), in both cases co-occurring with *H. pagellibogneravei*.

DISCUSSION

Infection levels

Infection levels in this study were higher than those previously reported, showing *Hatschekia pagellibogneravei* to be a common parasite of *Pagellus bogaraveo* off the coast of Madeira island. Although this parasite has been reported from blackspot seabream from the North Sea, it does not seem to be generally common in that region (Scott 1909). The highest prevalence reported for this parasite is 60%, detected in *Diplodus annularis* (Linnaeus), and the highest mean abundance (6.83) was detected in *P. bogaraveo* off Algeria, Mediterranean Sea (Boualleg et al. 2010a, b).

Copepods of the genus *Hatschekia* Poche, 1902 are more common in tropical and subtropical waters (Kabata 1979). Hermida et al. (2012) did not detect a single specimen of *H. pagellibogneravei* in a sample of 206 blackspot seabream off mainland Portugal, and only detected a prevalence of 5.8% in the Azores. Rohde and Heap (1998) found an increased abundance of ectoparasites in lower latitudes, possibly due to differences in water temperature range. The island of Madeira is located in a subtropical region, which might help explain the high abundance of this copepod in this region. The highest intensity observed in this study, 363 individuals on a single host, is remarkably high. However, it is not uncommon for species of this genus because Romero and Kuroki (1986) reported an intensity of 948 specimens of *Hatschekia affluens* Castro-Romero et Baeza-Kuroki, 1986 on the gills of *Pimelometopon maculatus* (Jenyns) from Chile. Although most species of *Hatschekia* produce clutches of less than 10 eggs, the observed specimens of *H. pagellibogneravei* had egg-strings of up to 22 eggs each, which is unusual for such a small copepod, and indicates a high reproductive effort (Kabata 1981).

Correlation between abundance and fish size

A negative correlation was found between *H. pagellibogneravei* abundance and fish size (both length and weight). Most studies of *Hatschekia* spp. have either failed to detect any correlation with fish size (Collins 1984, Scott-Holland et al. 2006) or have detected positive correlations (Grutter 1994, Geets et al. 1997, Lo et al. 1998). A positive correlation of parasite abundance with fish size would be expected in the case of parasites that accumulate on the host over time, and Geets et al. (1997) suggested that this might be the case of *Hatschekia* spp. However, Jones (1998), reviewing the literature on copepod life spans, concluded that they live from 2 months to about a year. Although very little is known about the life cycle of *Hatschekia* spp. (Bergh et al. 2001), there is no reason to suppose them to be living longer than other copepod species, thus limiting their accumulation on the host over time.

A correlation between *Hatschekia* spp. abundance and fish size might instead be related to differences in habitat of fish from different age groups. This seems to be the case of *Pagellus bogaraveo*, which exhibits ontogenetic changes of habitat, the juveniles preferring shallow coastal waters whereas adults inhabit deeper waters of up to 700 m (Morato et al. 2001). Water temperature rapidly decreases with depth below the surface layer, and it is known to be a limiting factor for parasitic copepods (Jones 1998). The preference for shallow waters on the part of younger blackspot seabream might thus increase their probability of coming into contact with the infective stages of *H. pagellibogneravei*. However, more information on the ecology and life cycle of this copepod is needed in order to validate that hypothesis.

Microhabitat distribution

There were no significant differences in abundance between the left and right gill. Most studies on the distribution of *Hatschekia* spp. also failed to detect any difference in abundance between the left and right sides of the body (Martens and Moens 1995, Geets et al. 1997, Muñoz and Cribb 2005, Scott-Holland et al. 2006), nor would any such differences be expected to occur in symmetrical fish. Collins (1984) found significant differences in abundance of *Hatschekia oblonga* Wilson, 1913 between the left and right gill of the host, *Ocyurus chrysurus* (Bloch), but concluded that this was probably due to chance, which is likely, considering the low number of individuals involved.

There were significant differences in the distribution of *H. pagellibogneravei* among the four gill arches. The fourth arch was significantly less parasitised, and a II-III-I-IV pattern of infection was observed, although differences between the first three arches were not statistically significant. Two patterns of distribution of *Hatschekia* spp. on the gills of their hosts have so far been reported, namely a preference for the central gill arches in detriment of arches 1 and 4 (Lo and Morand 2001, Scott-Holland et al. 2006), and a preference for the first two arches with a decline towards the fourth arch (Collins 1984, Martens and Moens 1995, Geets et al. 1997, Muñoz and Cribb 2005). In almost all cases, arch number 4 is the least parasitised, a finding which is confirmed in this study. Differences in gill arch preference have been explained in terms of differential volumes of water passing through the four gill arches (Llewellyn 1956, Scott-Holland et al. 2006). Llewellyn (1956) suggested that these differences in water flow would be reflected in differences in opportunities of parasites to attach. The distribution of parasites on the gills might simply reflect the relative probability of contact with a particular gill arch. However, it could also be the case that parasites attached to gills suffering from stronger water currents might have greater difficulty in remaining attached, and hence have lower survival rates.

Paling (1968) studied the relative volumes of water flow passing through the four gill arches of trout, *Salmo trutta* Linnaeus, by using glochidia larvae as marker parasites. These entered the mouth passively with the respiratory current and then attached to the gill filaments, distributing themselves over the gills in proportions reflecting the volume of water passing through each gill (Paling 1968). This author observed that a much larger volume of water passed through the two middle gill arches, and that the fourth gill arch is the less ventilated. A II-III-I-IV pattern of infection was observed by Paling (1968), which is the exact same pattern as that observed for *H. pagellibogneravei* in the present study. In fact, disregarding those parasites of uncertain provenance, the percentages of *H. pagellibogneravei* detected in each gill arch pair (24.6%, 33.7%, 28.0%, 13.7%) very closely resemble the proportions of glochidia larvae on the gills of trout (25.1%, 32.5%, 27.5%, and 14.9%, respectively) found by Paling (1968), suggesting that the distribution of *H. pagellibogneravei* is conditioned by differences in water flow within the gill habitat.

Other explanations that have been proposed to account for differences in microhabitat preferences of gill parasites include the effect of predators (Scott-Holland et al. 2006), interspecific competition and aggregation for increased mating opportunities (Rohde 1991). The effect of predators on *Hatschekia* spp. populations is poorly known; however, Scott-Holland et al. (2006) found that *H. plectropomi* did not seem to be particularly targeted by cleaner fish, suggesting that these fish probably select larger parasite species, overlooking these very small and relatively inconspicuous copepods. With regard to interspecific competition, there was relatively little contact of *H. pagellibogneravei* with other gill parasites of *P. bogaraveo* in the present study, so it was not possible to evaluate the effect, if any, of interspecific competition on microhabitat preferences. Timi (2003) evaluated the hypothesis of aggregation to facilitate mating in *Lernanthropus cynoscicola* Timi et Etchegoin, 1996 and rejected it, having found that aggregation among individuals of the same sex was stronger than among males and females. All *H. pagellibogneravei* specimens detected in the present study were female and, in fact, the male of this species is currently unknown; therefore, the data do not allow any speculation regarding the hypothesis of aggregation to facilitate mating.

Differences in water flow seem, in this case, to constitute the best explanation for the distribution pattern observed. It remains, however, unclear whether this distribution pattern results simply from differences in the probability of attachment, as suggested by Llewellyn (1956), or by an active preference of the copepod for better ventilated gills, for example due to specific respiratory requirements (Timi 2003). The fact that specimens of *Hatschekia* spp. seem to have the capacity to move on the gill habitat (Lo and Morand 2001) suggests that the distribution of these copepods on the gills of the host could indeed involve an active preference.

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CHAPTER 3

Endoparasites

ENDOPARASITES OF MARINE FISH

Marine fish can be hosts to a wide diversity of endoparasites. Endoparasite fauna is dependent on habitat and feeding habits, since most endoparasites are trophically acquired. In addition, most parasites show some specificity towards their hosts, which means that not all parasites available in a given region can successfully develop in any fish host. At the local level, parasite communities reflect the local availability of parasite species and their probability of colonization (Poulin 1997).

Most endoparasite species have complex life cycles, including at least an intermediate and a definitive host, and many species have more than one obligatory intermediate host. In addition, marine parasites often have the ability to incorporate paratenic hosts into their life cycles, which enables them to navigate the long and complex food webs that characterize the marine environment (Marcogliese 2002). Some acanthocephalans can even survive post-cyclic transmission, when a fish that would serve as an adequate definitive host is ingested by another fish on the trophic level above (Kennedy 2006).

Helminth communities in fish include a higher proportion of trematodes and acanthocephalans than communities in terrestrial vertebrates (Poulin & Leung 2011). Nematodes show the reverse trend, presenting much less diversity in fish when compared to terrestrial vertebrate taxa, suggesting a terrestrial origin of nematode parasitism (Anderson 1996). Marine fish, especially those that occupy a medium position in the food webs, can be simultaneously definitive and paratenic hosts to many different parasite species (Marcogliese 2002).

ANISAKID NEMATODES

Biology and ecology of anisakid nematodes

Anisakid nematodes are common marine parasites with a complex life cycle that includes euphausiacean crustaceans as obligatory intermediate hosts, fish or cephalopods as paratenic hosts, and, usually, marine mammals as definitive hosts (Køie 2001). Exceptions are *Hysterothylacium* spp., which have fish as definitive hosts, and some *Contracaecum* spp., which infect piscivorous birds. Anisakid nematodes typically show little specificity towards their paratenic hosts, but can be highly specific to the definitive host.

Within the widely distributed genus *Anisakis*, there are two phylogenetic clades, corresponding to two distinct larval morphotypes. Clade I includes *Anisakis simplex* sensu stricto, *A. pegreffii*, and *A. simplex* C, which together form the *Anisakis simplex* sensu latu complex, and also *A. typica*, *A. ziphidarum*, and *A. nascettii* (Mattiucci & Nascetti 2008, Mattiucci et al. 2009), whereas clade II includes the species *A. physeteris*, *A. brevispiculata* and *A. paggiae*. All *Anisakis* spp. infect cetaceans. Species belonging to the *A. simplex* complex can use several whales and dolphins as definitive hosts, whereas *A. ziphidarum* and the closely related *A. nascettii* are specific for beaked whales of the family Ziphiidae (Mattiucci & Nascetti 2008, Mattiucci et al. 2009). In the second clade, *A. physeteris* is specific for sperm whales (*Physeter macrocephalus*), and the other two species infect kogiid whales (Mattiucci & Nascetti 2008).

Anisakid nematodes, and in particular *Anisakis* spp., can accidentally infect humans through the ingestion of raw or undercooked fish, causing anisakiasis, which involves a variety of gastrointestinal symptoms, allergic reactions, or both (Deschner et al. 2000).

Since these parasites remain in the host fish for long periods of time, they are particularly appropriate as biological tags, and have been successfully used towards this end in the study of fish populations (e.g. MacKenzie et al. 2008). Their biogeographical distribution patterns are mostly related to differences in the distribution of their definitive hosts.

Identification of anisakid larvae parasitic in fish

Anisakid nematodes occur in fish as larvae; and as such are extremely difficult, and in some cases impossible, to identify morphologically. Furthermore, several cryptic species have been discovered in recent years through the use of molecular techniques, which have become the only reliable way to identify these nematodes. Several molecular approaches can be used for anisakid nematode identification, including allozyme electrophoresis, PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) or PCR-SSCP (single-strand conformation polymorphism) of the ITS region of rDNA, PCR using specific primers, and direct sequencing of nuclear (ITS, 28S) or mitochondrial (*cyt-b*, *cox2*) markers.

Allozyme electrophoresis was the first molecular method to be applied to the identification of anisakid parasites of fish (Mattiucci & Nascetti 2008), but as the technology evolves, protein-based methods tend to be replaced by DNA-based ones, which can detect differences that are often masked in end-products.

PCR-RFLP of the ITS1-5.8S-ITS2 region of rDNA was proposed as a method of identifying anisakid nematodes parasitizing fish by Zhu et al. (1998), and has since been widely used for this purpose. PCR-RFLP is a method that detects differences in nucleotide sequences without the need to painstakingly sequence every single specimen, by means of digestion with restriction enzymes that cut in specific places. Once the genetic sequence of each species is known, a simulation of restriction sites for a number of enzymes is performed, in order to select the more adequate enzymes to accurately distinguish between species. The PCR products are digested with the selected endonucleases and the digestion products are then run on an agarose gel, where distinct patterns of bands appear.

The ITS (internal transcribed spacer) region of rDNA is particularly useful as a genetic marker for this kind of study because it includes two non-coding regions, ITS1 and ITS2, which are invisible to natural selection and therefore accumulate mutations at a much higher rate than functional genes. These are flanked by highly conserved, functional genes, which enable the annealing of non-specific primers that can be used for different species.

Anisakid nematodes of the blackspot seabream

In the third paper (Hermida M., Mota R., Pacheco C.C., Santos C.L., Cruz C., Saraiva A., Tamagnini P. (2012) Infection levels and diversity of anisakid nematodes in blackspot seabream, *Pagellus bogaraveo*, from Portuguese waters, *Parasitology Research*, 110: 1919-1928), anisakid nematodes detected in *P. bogaraveo* were identified using PCR-RFLP and/or sequencing of the ITS region, and a phylogenetic analysis of the detected species, along with other sequences available from GenBank, was performed. Supplementary material to this paper is presented in appendix B.

A wide diversity of anisakid nematodes was detected, most of them for the first time, in *P. bogaraveo*. There were significant differences between regions both in terms of the relative abundance of the different species detected (Fig. 8), and also in anisakid infection levels. Mainland Portuguese waters were characterized by very high levels of infection by *Anisakis* spp. (Fig. 9), and almost all of the molecularly identified larvae from this region belonged to the *A. simplex* complex. These included *A. simplex* s.s., *A. pegreffii*, and hybrid genotypes of the two. Blackspot seabream from the Azores were characterized by low infection levels of anisakids, and the dominant species in that region was *A. physeteris*, a specific parasite of sperm whales (*Physeter macrocephalus*), which are common in that region. Madeira had the highest diversity of anisakid nematodes, with eight species detected. The dominant species in that region was the yet undescribed *Anisakis* sp. PB-2010, which sequence analysis showed to be most probably conspecific with *Anisakis* sp. HC-2005, detected in fish from the African shelf (Kijewska et al. 2009). In fact, the region of Madeira and the African continental shelf at similar latitudes seem to be a hotspot of *Anisakis* spp. diversity, including not only several well-known species but also others that have yet to be properly described. A geographical summary of the species of *Anisakis* detected in teleost fish from the north-east Atlantic is presented in Fig. 10.

GASTROINTESTINAL HELMINTH COMMUNITIES

Gastrointestinal parasites of fish

Unlike anisakid nematodes and other parasites that occur in fish as juveniles and are most commonly located in the body cavity or encysted in the mesenteries, parasites occurring in the gastrointestinal tract of fish in the vast majority have fish as their definitive hosts. Gastrointestinal helminth communities of marine teleost hosts are most commonly dominated by digeneans (Kennedy 2006), but they also usually include varying proportions of acanthocephalans, nematodes, and cestodes.

The gastrointestinal tracts of fish are very variable, having followed different evolutionary routes according to the lifestyle and feeding habits of the fish. For the parasites that inhabit them, the gastrointestinal tract is a complex environment, with very different microhabitat conditions prevailing in different subsections (Crompton 1970). Many helminth species have a restricted niche, occupying only certain portions of the gastrointestinal tract. Hemiurid digeneans, for example, are more or less restricted to teleost stomachs, and have evolved a thick tegument and retractile ecsoma as protection in that acidic environment (Gibson & Bray 1986). Microhabitat distribution, in turn, is important in determining the structure of parasite communities (Sasal et al. 1999).

The gastrointestinal helminth communities of *P. bogaraveo*

In the fourth paper (Hermida M., Cruz C., Saraiva A. Gastrointestinal helminth communities of the blackspot seabream *Pagellus bogaraveo* (Teleostei: Sparidae) from Portuguese north-east Atlantic waters, *Journal of Helminthology*, *in press*), the gastrointestinal helminth communities of blackspot seabream were analysed and community properties ascertained through the use of ecological indices. Fifteen helminth species were detected, including several new records in this host. Infection levels were generally low. The zoogonid digeneans *Diphterostomum vividum* (Fig. 11) and *Brachyenteron helicoleni* (Fig. 12) had the highest abundance and dominated all communities with the exception of the Azores, where the acanthocephalan *Rhadinorhynchus pristis* (Fig. 13) was dominant. *Brachyenteron helicoleni* has been recently described in *Helicolenus dactylopterus* off Scotland (Bray & Kutcha 2006) and is reported here for the first time in another definitive host.

Blackspot seabream from mainland waters had much higher infection levels, especially of digenean species. The gastrointestinal helminth community of *P. bogaraveo* from Madeira was exceedingly depauperate, whereas in the Azores diversity was relatively high, but infection levels were also very low.

Several species exhibited microhabitat preferences within the gastrointestinal tract of the host. Hemiurids such as *Lecithocladium excisum* (Fig. 14) were mostly found in the stomach, and zoogonids in the posterior part of the intestine. The distribution of organisms in niche space is an important factor in determining community properties, and Keeney & Poulin (2007) have recently applied the concepts of functional richness and functional evenness to parasite communities, using as an example the distribution of cestode parasites in the spiral intestines of elasmobranch hosts. Functional richness is a measure of the proportion of niche space that is occupied, whereas functional evenness measures the evenness in the distribution of organisms along niche space.

Here these concepts are applied to the whole gastrointestinal helminth community of a teleost fish for the first time, and results show that they capture important properties of parasite communities, complementing other more commonly used ecological indices. Taken together these indices can indicate whether resources are being underutilized.

In the case of blackspot seabream gastrointestinal helminth communities, fish from mainland waters had both higher functional richness and functional evenness, indicating more stable communities, whereas in Madeira the very impoverished helminth community also presented low functional richness and, in the Azores, even though the community was diverse, infection levels were very low, and a low functional evenness was observed. The differences detected in parasite communities from these three regions are likely to reflect biogeographical differences in the availability of benthic invertebrates that can be intermediate hosts of digeneans, and also a shift to a more pelagic diet in subtropical waters.

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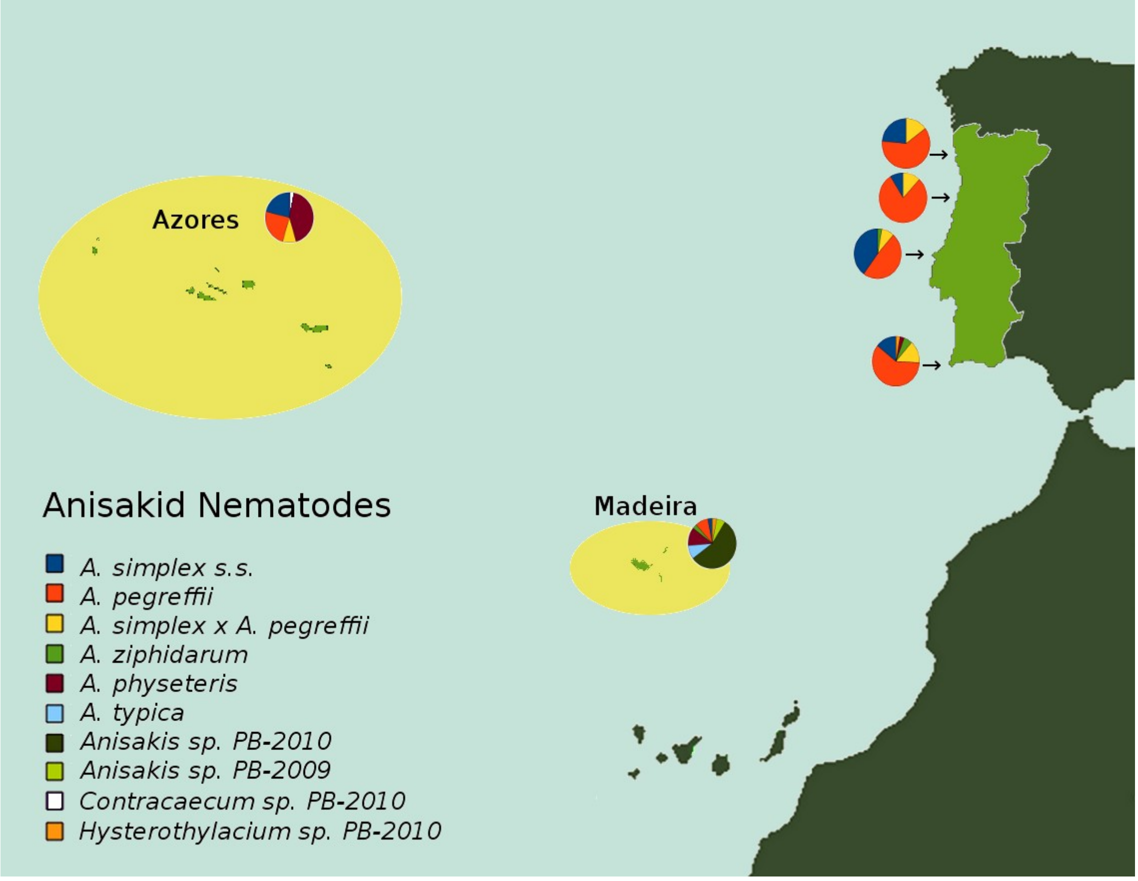

Fig. 8. Relative abundance of anisakid nematodes detected in *Pagellus bogaraveo* in this study.

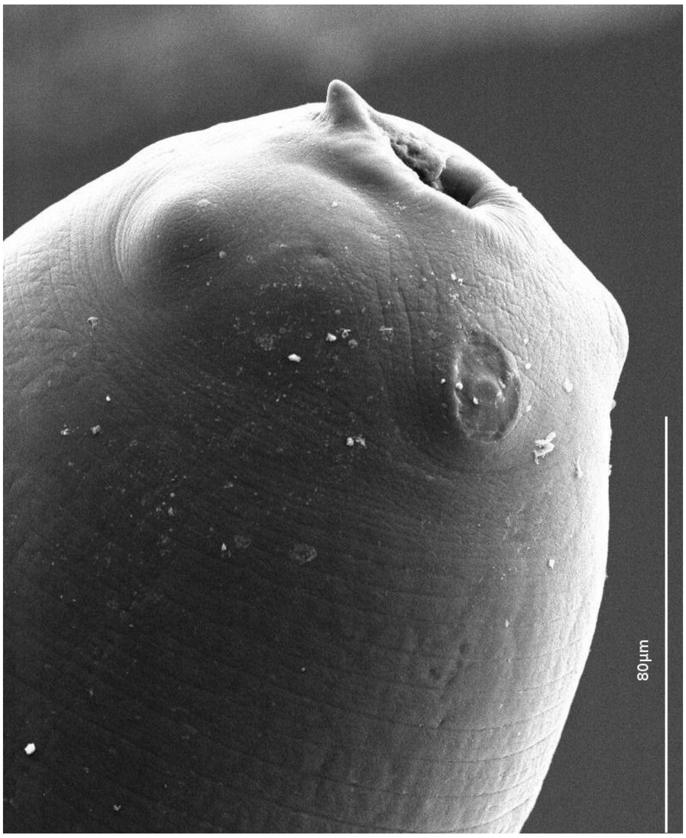

Fig. 9. *Anisakis* sp. (Nematoda: Anisakidae), anterior part.

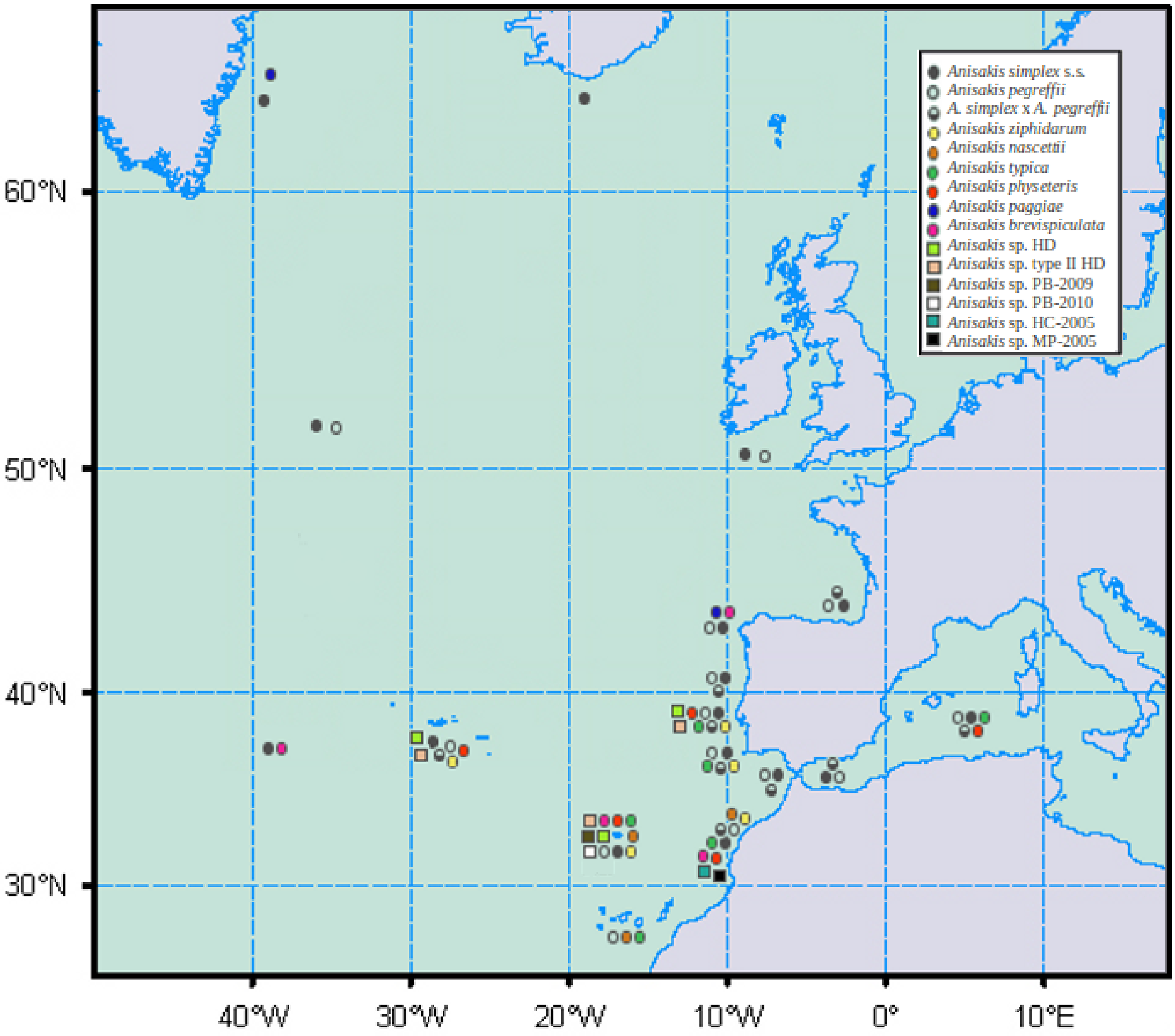

Fig. 10. *Anisakis* spp. molecularly identified from fish in the north-east Atlantic region (squares represent undescribed species).

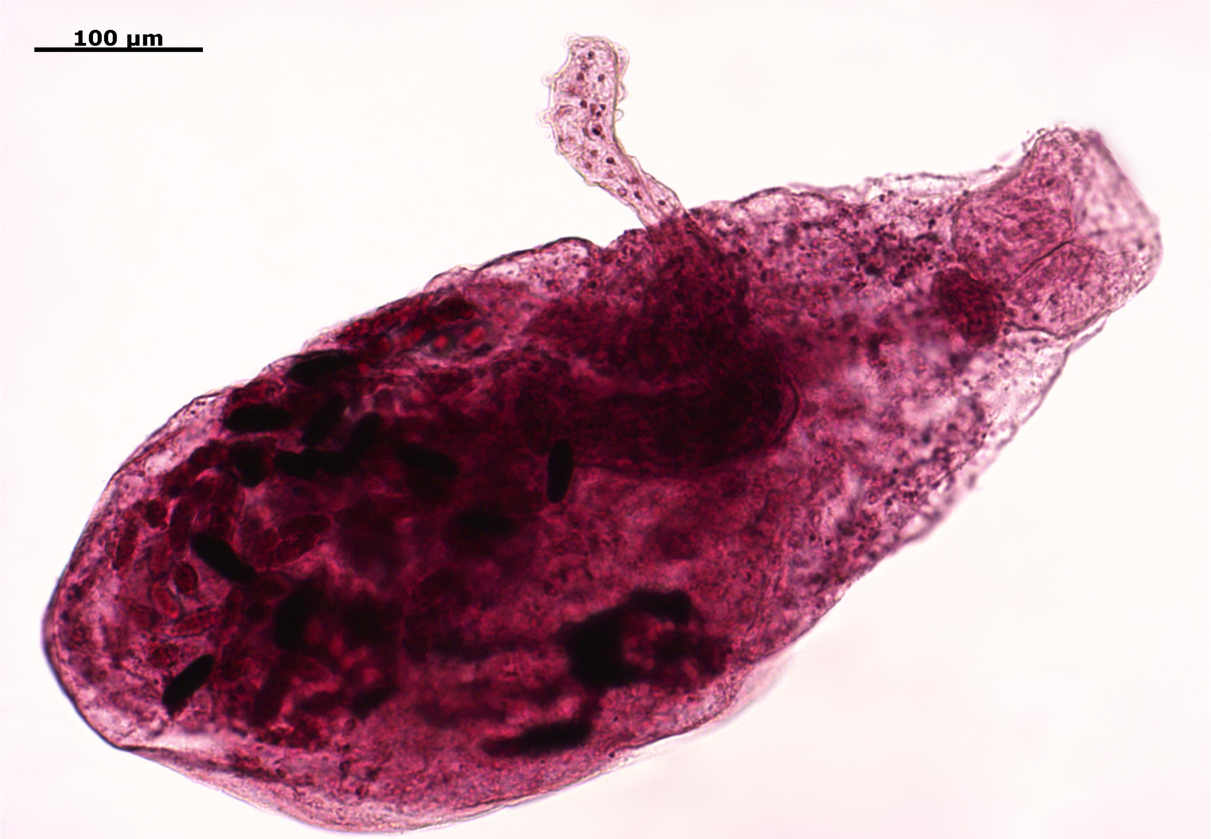

Fig. 11. *Diphterostomum vividum* (Digenea: Zoogonidae).

Fig. 12. *Brachyenteron helicoleni* (Digenea: Zoogonidae).

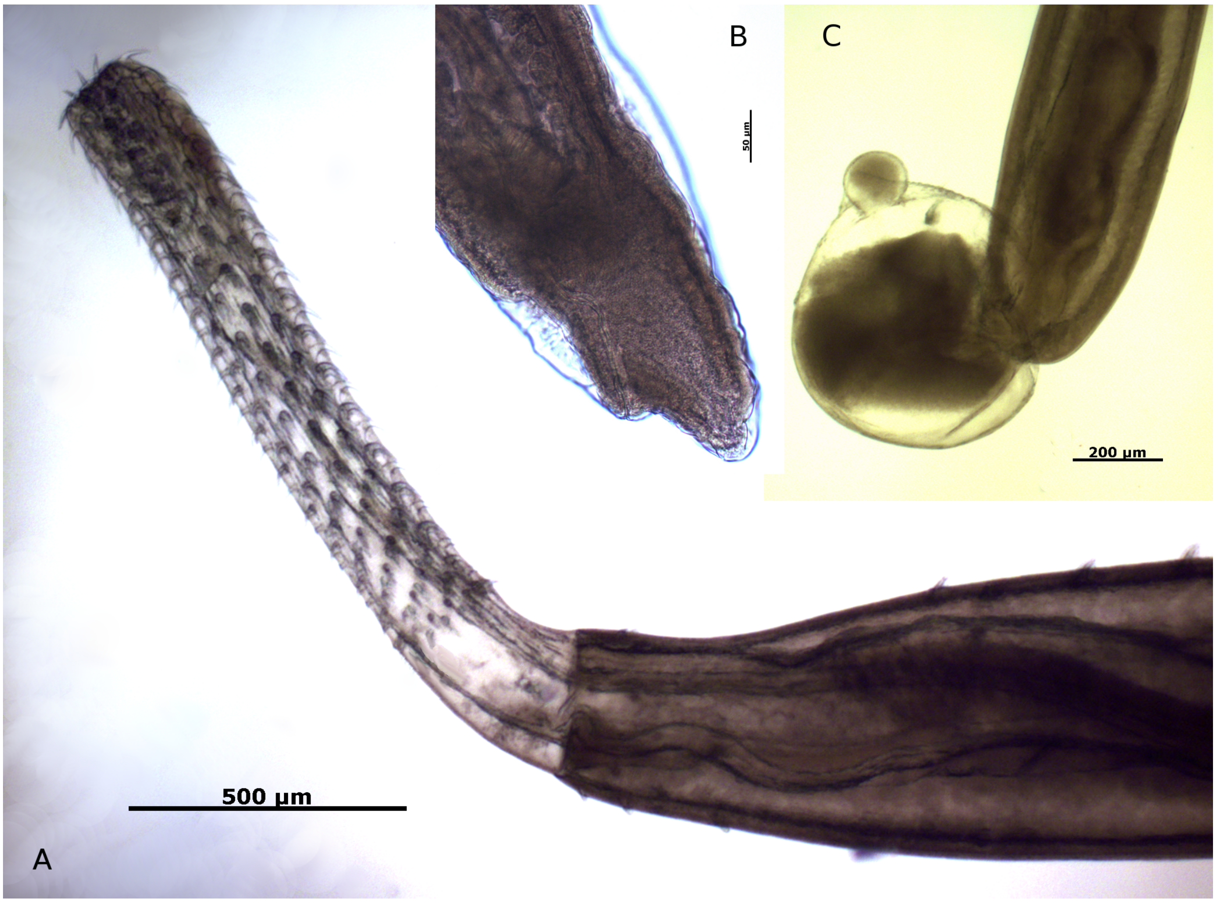

Fig. 13. *Rhadinorhynchus pristis* (Acanthocephala: Rhadinorhynchidae). A, proboscis; B, posterior part of female; C, posterior part of male.

Fig. 14. *Lecithocladium excisum* (Digenea: Hemiuridae).

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ORIGINAL PAPER

Infection levels and diversity of anisakid nematodes in blackspot seabream, *Pagellus bogaraveo*, from Portuguese waters

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Abstract The blackspot seabream, *Pagellus bogaraveo*, is a sparid fish of great economic importance in the northeast Atlantic. The main aim of this work was to assess the infection levels and diversity of anisakid nematodes parasitizing *P. bogaraveo* from Portuguese waters. The anisakid larvae were identified by polymerase chain reaction-restriction fragment length polymorphism analysis and ten different patterns were observed, four of which were not previously reported in the literature. Moreover, several species were detected for the first time in this host: *Anisakis simplex*×*Anisakis pegreffii* hybrids, *Anisakis ziphidarum*, *Anisakis typica*, *Anisakis physeteri*s, as well as three undescribed anisakids *Anisakis* sp. PB-2009, *Anisakis* sp. PB-2010, and *Contracaecum* sp. PB-2010. The ITS1-5.8S-ITS2 region was sequenced and analyzed phylogenetically, revealing that our anisakids were distributed by the two distinct clades reported previously, corresponding to the two recognized larval morphotypes. Moreover, a group of organisms, including our specimens from Madeira and the previously reported *Anisakis* sp. HC-2005, cluster together and seem to belong to clade I. A certain degree of intraspecific diversity was also detected. Samples from mainland waters had the highest infection levels and were dominated by *A. pegreffii*. Madeira had the highest diversity overall, dominated by *Anisakis* sp. PB-2010. Fish from the Azores had the lowest infection levels, and the species with the highest relative abundance was *A. physeteris*. The anisakid nematode communities were relatively similar in mainland waters but very distinct in both the Azores and Madeira islands, suggesting the existence of at least three different stocks of *P. bogaraveo* in the northeast Atlantic.

Introduction

The blackspot seabream, *Pagellus bogaraveo* (Brünnich, 1768), is a greatly appreciated fish with high commercial value. It is a sparid fish whose geographical distribution encompasses the Northeast Atlantic and the Mediterranean, occurring at depths of up to 800 m (Spedicato et al. 2002). It is a protandrous hermaphroditic species, with most individuals maturing first as males and later changing sex to females (Micale et al. 2002). This feature seems to increase the species' vulnerability to fishing pressure, as evidenced by the collapse of the Cantabrian Sea *P. bogaraveo* stock (Erzini et al. 2006). Little is known about its population structure in the northeast Atlantic, although several studies seem to point to low genetic variability (Stockley et al. 2005; Lemos et al. 2006). Most of the fishery catches of this species are from Portugal and Spain, and recently, this fish

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has been introduced into aquaculture (Basurco et al. 2011). Recent shifts in gastronomic habits, especially the consumption of raw fish, have contributed to an increased awareness of fish parasites as a health risk. Anisakid larvae, especially *Anisakis* spp., can infect humans and cause anisakiosis, a condition that includes a number of mild to severe gastrointestinal problems, as well as acute allergic reactions (Audicana et al. 2002; Falcão et al. 2008; Hochberg and Hamer 2010). Recently, *A. simplex* extracts have been included in the standard sets of allergens for the evaluation of food allergies (Audicana and Kennedy 2008).

Parasites have been widely used as biological tags to study fish stocks, and anisakid nematodes are especially suited for this purpose because once acquired they tend to remain in the host fish for long periods of time (MacKenzie and Abaunza 1998). However, as fish are generally intermediate or paratenic hosts of these parasites (with marine mammals as definitive hosts), only larvae occur in fish, exhibiting little morphological differences, which hinders their identification. Some formerly recognized species have been shown to be complexes of cryptic species, e.g., *A. simplex sensu lato* (s.l.), with genetic but not morphological differentiation (Mattiucci and Nascetti 2008). Molecular markers, in particular, the rDNA region spanning the internal transcribed spacers ITS1 and ITS2, and the 5.8S subunit are widely used in taxonomic studies of nematodes and have been an invaluable tool to discriminate among several closely related anisakid species (Mattiucci and Nascetti 2008).

In the northeast Atlantic, several species of the genus *Anisakis* have been reported in teleosts. At high latitudes, *A. simplex* s.s. is often the only species found in these hosts (e.g., Klimpel et al. 2007, 2008). Recently, Klimpel et al. (2011) have also detected *Anisakis paggiae* in a deep-sea fish at latitudes of 52°–58°N. In general, *A. simplex* s.s. becomes less abundant towards the south. *Anisakis pegreffii* is the most common anisakid in the Mediterranean Sea (e.g., Abollo et al. 2003; Mattiucci et al. 2004; Martín-Sánchez et al. 2005) and has been detected in Atlantic areas with Mediterranean influence. In sympatric areas of *A. simplex* s.s. and *A. pegreffii*, hybrid genotypes have been detected, namely in the Cantabrian Sea, Portuguese mainland coast, Gibraltar area, and the Azores (Abollo et al. 2003; Martín-Sánchez et al. 2005; Marques et al. 2006; Sequeira et al. 2010). In Portuguese waters, several other *Anisakis* species have also been detected, including *Anisakis typica*, *Anisakis physeteris*, and *Anisakis ziphidarum*, the latter only in islands' waters (Marques et al. 2006; Sequeira et al. 2010). Anisakid diversity seems to increase towards lower latitudes, and, around Madeira, several species occur, including *A. simplex* s.s., *A. pegreffii*, *A. ziphidarum*, *A. typica*, *A. physeteris*, *Anisakis brevispiculata*, and *Anisakis nascettii* (Costa et al. 2004; Pontes et al. 2005; Sequeira et al. 2010). The distribution of anisakid species is undoubtedly related to the presence of their definitive hosts and conditioned by the local availability of suitable intermediate and paratenic hosts (Klimpel et al. 2010).

The main aim of this work was to determine the infection levels of *P. bogaraveo* by anisakid nematodes, as well as to assess their diversity at different locations in Portuguese waters by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and direct sequencing of the internal transcribed spacer (ITS) region.

Material and methods

Fish sampling and parasitological analysis

A total of 269 specimens of *P. bogaraveo* from Portugal Exclusive Economic Zone, namely Matosinhos (42), Figueira da Foz (52), Peniche (42), Sagres (30), Madeira (36), and the Azores (67) (see Fig. 1) were acquired from commercial catches. The fish were transported in ice, placed in individual plastic bags, and frozen until further observation. After defrosting, specimens were measured, dissected, and examined for the presence of anisakid larvae in the body cavity, viscera, and mesenteries, under a stereomicroscope. All anisakid larvae detected were removed, counted, washed in physiological saline 0.9% (*p/v*) and preserved in 70% (*v/v*) ethanol. For each location, infection levels of anisakid larvae (prevalence, intensity, and abundance) were determined following Bush et al. (1997).

DNA extraction, PCR-RFLP, and sequencing

DNA was extracted by crushing and grinding the anisakid larva with a sterile pestle in an Eppendorf tube containing 20 μl of buffer (50 mM Tris–HCl, pH 8.0, with 10 mM EDTA) and using the QIAamp DNA MiniKit (QIAGEN, Germany) following the protocol for DNA extraction from tissues. The DNA was eluted with 20–30 μl of water.

Polymerase chain reaction (PCR) amplification of the ITS region was performed using the NC5 forward (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 reverse (5′-TTAGTTTCTTCCTCCGCT-3′) oligonucleotide primers (Zhu et al. 1998). PCRs were carried out in a MyCyclerTM thermal cycler (Bio-Rad laboratories Inc., Hercules, CA, USA) using 0.5 U of Taq polymerase and 1× PCR reaction buffer supplemented with 1 mM MgCl2 (GE Healthcare, Buckinghamshire, UK), 200 mM deoxynucleoside triphosphates, 1 mM of each primer, 16% (*p/v*) of bovine serum albumin and 50 to 250 ng of DNA, with the following program—94°C for 10 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 70s, and a final extension of 72°C for 7 min. Agarose gel electrophoresis

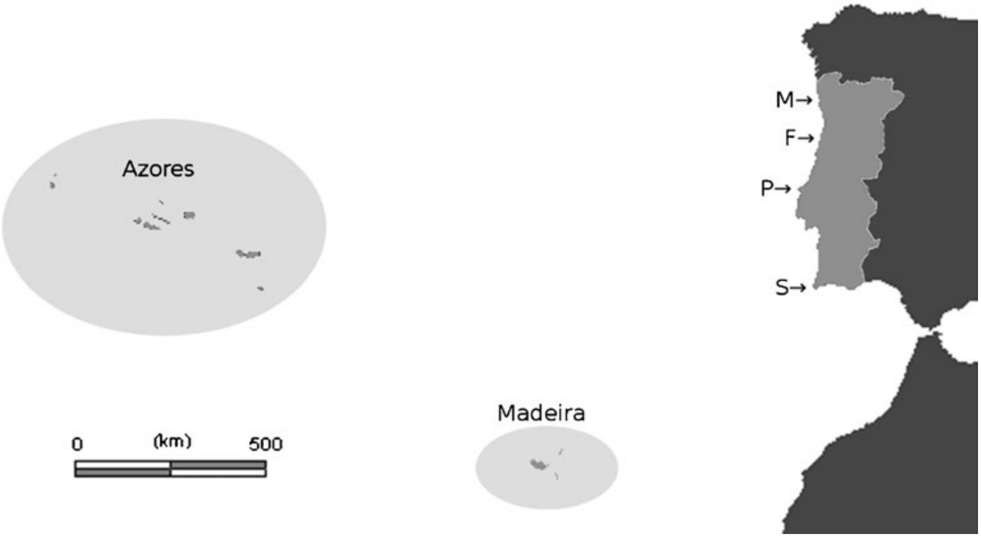

Fig. 1 Sampling locations in Portuguese waters (northeast Atlantic); *M*, Matosinhos; *F*, Figueira da Foz; *P*, Peniche; *S*, Sagres; Madeira; and the Azores islands

was performed with standard protocols using 1× TAE buffer (Sambrook and Russell 2001).

At least 30 larvae were randomly selected among the total sample from each location for the molecular identification in order to detect, with a 95% confidence level, all the species present with a relative abundance$\geq$10% (Simon and Schill 1984). After PCR, about 200 ng of the ITS amplicon (~1 kb) was digested with the restriction enzyme FastDigest® *Hin*fI (Fermentas, York, UK), according to manufacturer instructions. When further discrimination was necessary, the endonucleases FastDigest® *Hha*I and/or *Taq*I were also used. The RFLP patterns were evaluated by electrophoresis using 2–2.5% ethidium-bromide-stained agarose gels.

The DNA fragments were isolated from agarose gels using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). Purified PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5α competent cells following the instructions of the manufacturer. White colonies were grown overnight at 37°C, in liquid LB medium supplemented with 100 μg ml^{-1} of ampicillin, with shaking. Plasmid DNA was isolated from *E. coli* cultures using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Saint Louis, MO, USA), and the insert was sequenced at STAB Vida (Lisbon, Portugal). Novel sequences associated with this study were deposited in GenBank (Accession numbers: JN005754 to JN005769).

Phylogenetic analysis

The ITS1-5.8S-ITS2 sequences from the anisakid species isolated in this study were aligned with a series of *Anisakis* spp. reference species and *Pseudoterranova decipiens*, using the ClustalW (Larkin et al. 2007) plug-in present in the Geneious Pro package (Drummond et al. 2011). This alignment was used to build a neighbor-joining (NJ) phylogenetic tree (Saitou and Nei 1987) using *P. decipiens* as the outgroup. This tree was computed using the MEGA5 software (Tamura et al. 2011), considering the Kimura-2 parameter model (Kimura 1980) with a rate variation among sites modeled with a gamma distribution (as selected by the Model Selection tool built in MEGA). For the bootstrap analysis, 1,000 pseudo-replicates were used (Felsenstein 1985).

Statistical analysis

Statistical analyses were carried out using IBM® SPSS® Statistics software. The nonparametric Mann–Whitney *U* test was used to perform a pairwise comparison of fish length and anisakid abundance between locations (differences were considered significant at $p<0.05$). The relation between fish length and anisakid abundance was analyzed by Spearman rank correlation. A pairwise analysis of the relative abundance of the identified anisakid species was carried out between locations using Chi-square test (differences were considered significant at $p<0.05$).

Results

Infection levels

Most anisakid larvae were found encapsulated in flat coils in the fish body cavity, mesenteries, and on the outside of viscera, sometimes forming large clusters. Some larvae were free in the body cavity and, occasionally, inside visceral organs.

Fish length and infection levels are presented in Table 1. Fish length varied significantly among locations, with fish from Madeira and Sagres being the smallest and the ones from Peniche being the largest. Most of the sampled fish were infected, with prevalence reaching 100% in Peniche and Matosinhos. The fish from Azores were the only exception with a prevalence of 36%. Abundance of infection varied significantly among locations, showing very low values in the Azores, low values in Sagres, and high values in Peniche. A significant positive correlation was found between anisakid abundance and fish length ($r=0.273$, $n=268$, $p=0.000$).

Identification and geographical distribution of anisakid species

Two hundred five anisakid larvae isolated from *P. bogaraveo* (at least 30 randomly selected from the total sample from each location, see section "Material and methods") were identified by PCR-RFLP pattern analysis. Ten different patterns were observed (Table 2). Five patterns were previously described (D'Amelio et al. 2000), corresponding to the species *A. simplex* s.s., *A. pegreffii*, *A. ziphidarum*, *A. physeteris*, and *A. typica* (Table 2 and Electronic supplementary material Fig. S1: lanes 1, 2, 4, 5, 6, respectively). One pattern consisting of the combined patterns of both *A. simplex* s.s. and *A. pegreffii* was found, corresponding to the recombinant genotype first described by Abollo et al. (2003) (Table 2 and Electronic supplementary material Fig. S1: lane 3). Four novel patterns were also detected. For most of the Madeira specimens, the 869-bp PCR fragment was not cut by *Hin*fI, and since this pattern is typical for both the species, *A. paggiae* and *A. brevispiculata* (Quiazon et al. 2009; Murata et al. 2011; Cavallero et al. 2011), the fragment was subjected to digestion with *Hha*I and *Taq*I (Table 2 and Electronic supplementary material Fig. S1: lane 7). However, the patterns obtained did not correspond to either *A. paggiae* (Cavallero et al. 2011; Murata et al. 2011) or *A. brevispiculata* (Sequeira et al. 2010; Murata et al. 2011; Cavallero et al. 2011). Subsequently, the PCR fragment obtained for *Anisakis* sp. PB-2010 was cloned and sequenced (JN005761), and it was found to have 99% similarity to *Anisakis* sp. HC-2005 in *Hoplostethus cadenati* from the African shelf (Kijewska et al. 2009). In two specimens from Madeira, *Anisakis* sp. PB-2009, a different pattern was observed (Table 2), and their sequences (JN005758 and JN005759) had 94% similarity to the above-mentioned *Anisakis* sp. HC-2005 as well as *Anisakis* sp. PB-2010. Two other unknown patterns (Table 2 and Electronic supplementary material Fig. S1, lanes 8 and 9) were also found and the respective fragments sequenced: One of the sequences (JN005769) showed 98% similarity to *Contracaecum* sp. 2 SX-2008 (Fang et al. 2010), a sequence that in fact is more similar to several *Hysterothylacium* spp. than to any *Contracaecum* spp., and therefore our specimen was identified as *Hysterothylacium* sp. PB-2010, whereas the other (JN005755) has 88% similarity to *Contracaecum muraenesoxi* (Luo 1999; Fang et al. 2010) and was identified as *Contracaecum* sp. PB-2010.

The number of anisakid nematodes from each species, their relative abundances, and statistical significance of differences among locations is presented in Table 3. The relative abundances of anisakid species in each sampling location are depicted in Fig. 2.

In the samples from mainland waters, the relative abundance of larvae belonging to the *A. simplex* complex was very high (between 89% in Sagres and 100% in Figueira da Foz and Matosinhos), whereas in the Azores it was considerably lower (55%), reaching only 12% in Madeira. *A. pegreffii* was the most abundant species in all the mainland samples, whereas in the Azores *A. physeteris* presented the highest relative abundance (42%), and in Madeira *Anisakis* sp. PB-2010 was the dominant species with a relative abundance of 56%. This species, as well as the closely related *Anisakis* sp. PB-2009, only occurred in Madeira, and the larvae were mostly encysted in the stomach wall (69%).

Anisakid species richness decreased with latitude, with the highest diversity in Madeira (eight species) and lowest in

Table 1 Number of fish examined (*N*), fish total length, anisakid infection levels (prevalence, intensity, and abundance) in each sampling location and significance of differences between locations (similar letters indicate no statistically significant differences at $p<0.05$)

Location	*N*	Fish total length (cm)			Prevalence	Intensity		Abundance		
		Mean±SD (min–max)	Median		(%)	Mean±SD (min–max)	Median	Mean±SD	Median	
Matosinhos	42	32.1 ±1.7 (27.1-35.0)	32.4	b	100	12.6±11.9 (1–48)	8.5	12.6±11.9	8.5	d
Figueira da Foz	52	32.7±1.5 (28.8-37.6)	32.7	b	92	10.5±11.9 (1–49)	6	9.7±11.8	6	cd
Peniche	42	37.4±3.1 (33.2-48.2)	36.6	c	100	43.4±82.9 (1–449)	18	43.4±82.9	18	e
Sagres	30	30.0±0.7 (28.2-31.1)	30.1	a	73	9.1±15.9 (1–76)	4.5	6.7±14.2	2	b
Madeira	36	30.2±1.7 (27.6-34.2)	30.1	a	83	14.9±26.3 (1–125)	5.5	12.4±24.6	5	bc
Azores	67	33.5±4.1 (27.0-43.3)	32.5	b	36	2.67±2.62 (1–10)	2.6	1.0±2.0	0	a

Table 2 ITS region PCR product and PCR-RFLP fragment sizes obtained for the anisakids identified in this study

Species	PCR product (bp)	Fragment sizes (bp)		
		*Hin*fI	*Hha*I	*Taq*I
A. simplex s.s.	951	615, 232, *67, 37*	530, 421	424, 378, *65, 54, 30*
A. pegreffii	951	331, 284, 232, *67, 37*	530, 421	378, 293, 131, *65, 54, 30*
A. simplex s.s.×*A. pegreffii*	951	615, 331, 284, 232, *67, 37*	530, 421	424, 378, 293, 131, *65, 54, 30*
A. ziphidarum	929	332, (287, 273), *37*	516, 413	(285, 282), 128, *65, 54, 30*
A. typica	954	594, 326, *34*	308, 212, 180, 153, 101	380, 338, *65, 54, 28*
Anisakis sp. PB-2009	896	578, 284, *34*	526, 370	293, 171, 125, 91, *65, 54, 40, 28, 21, 8*
Anisakis sp. PB-2010	869	869	372, 211, 172, 114	391, 171, *65, 54, 40, 30, 21, 8*
A. physeteris	898	360, 260, 241, *37*	511, 387	272, 242, 133, *65, 54, 30, 12*
Hysterothylacium sp. PB-2010	1,033	685, 349	307, 271, 184, 150, *74, 48*	324, 298, 147, 118, *65, 54, 28*
Contracaecum sp. PB-2010	953	422, 339, 192	349, 306, 150, (*77, 71*)	306, 142, (112, 107), *65, 54, 46, 45, 41, 30, 5*

Fragments that appear as a single band are indicated in brackets. Fragments in italics might not be visible in the gel

Matosinhos and Figueira da Foz, where only *A. simplex* s.s., *A. pegreffii*, and hybrid genotypes between these two species were found.

Phylogenetic analysis

Phylogenetic analyses were performed to assess the relative positioning of the anisakid species identified in this study. A NJ algorithm was used to compute a phylogenetic tree of all isolates and a number of reference species, using *P. decipiens* as the outgroup. The resulting tree (Fig. 3) revealed the two distinct clades previously identified by a number of different authors (e.g., Mattiucci and Nascetti 2008). Moreover, we were able to identify a group of specimens from Madeira (*Anisakis* sp. PB-2009 and *Anisakis* sp. PB-2010) that cluster together with a species temporarily described as *Anisakis* sp. HC-2005. This group of organisms (Fig. 3, dashed line) seem to belong to clade I, with a high bootstrap (100%) support. In order to validate these phylogenetic results, a maximum-likelihood tree was also constructed (data not shown). This tree fully supported the topology of the NJ one (Fig. 3), both in the clade separation and in the relationship between clade I and the undescribed species.

Most species showed some degree of intraspecific variability, as can be seen in the estimates of evolutionary divergence and their standard errors (Table S1 of the Electronic supplementary material). Of the four *A. simplex* s.s. sequenced, JN005764 and EU624342 were identical, whereas JN005757 differ from JN005764 and EU624342 0.002 base substitutions per site. On the other hand, within

Table 3 Number and relative abundance (percent) of anisakid species detected in the different locations and significance of differences between locations

Anisakid species	Matosinhos (34)			Figueira da Foz (34)			Peniche (35)			Sagres (35)			Madeira (34)			Azores (33)		
	No.	%		No.	%		No.	%		No.	%		No.	%		No.	%	
A. simplex s.l.	34	100	c	34	100	c	34	97	c	31	89	c	4	12	a	18	55	b
A. simplex s.s.	8	23	ab	3	9	a	14	40	b	5	14	a	1	3	a	7	22	ab
A. pegreffii	21	62	bc	27	79	c	17	48	ab	21	60	bc	3	9	a	8	24	a
A. simplex×*A. pegreffii*	5	15	–	4	12	–	3	9	–	5	14	–	0	0	–	3	9	–
A. ziphidarum	0	0	–	0	0	–	1	3	–	2	6	–	1	3	–	0	0	–
A. typica	0	0	–	0	0	–	0	0	–	0	0	–	3	9	–	0	0	–
Anisakis sp.	0	0	a	0	0	a	0	0	a	0	0	a	21	61	b	0	0	a
Anisakis sp. PB-2009	0	0	–	0	0	–	0	0	–	0	0	–	2	6	–	0	0	–
Anisakis sp. PB-2010	0	0	a	0	0	a	0	0	a	0	0	a	19	55	b	0	0	a
A. physeteris	0	0	a	0	0	a	0	0	a	1	3	a	4	12	a	14	42	b
Hysterothylacium sp. PB-2010	0	0	–	0	0	–	0	0	–	1	3	–	1	3	–	0	0	–
Contracaecum sp. PB-2010	0	0	–	0	0	–	0	0	–	0	0	–	0	0	–	1	3	–

Similar letters indicate no statistically significant differences at $p<0.05$

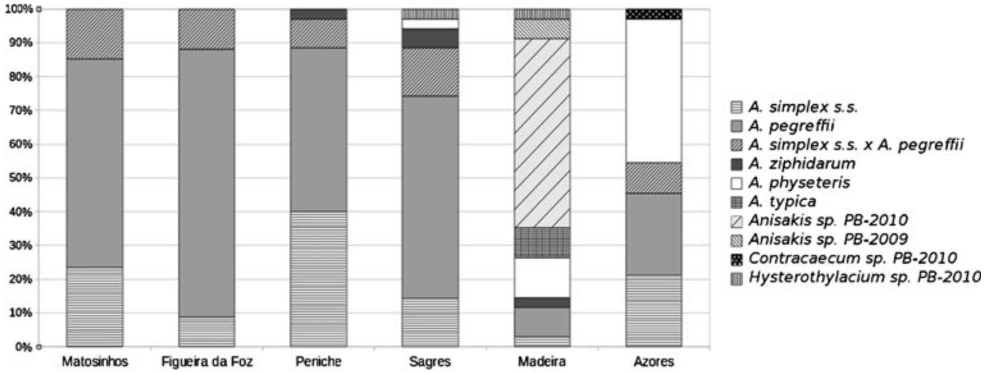

Fig. 2 Relative abundances of anisakid species in each sampling location

the species *A. pegreffii*, the distance ranged between 0.001 and 0.006. The two consistent differences that have been reported between *A. simplex* s.s. and *A. pegreffii* in positions 280 and 296 of the ITS-1 regions, T in *A. simplex s.s.* and C in *A. pegreffii* (Abollo et al. 2003; Ceballos-Mendiola et al. 2010; Du et al. 2010), were also detected in our sequences. However, a hybrid sequence (JN005768), which had a C at position 280 but a T at position 296 of the ITS-1 region, is reported here for the first time. The two *A. ziphidarum* sequences from Sagres have a 0.005 phylogenetic distance. *A. physeteris* sequences showed a maximum difference between two individuals of 0.008 base substitutions per site. In contrast, *A. typica* were only separated by a maximum of 0.002, whereas *Anisakis* sp. PB-2009 0.003.

Discussion

In this study, a wide diversity of anisakids was detected in *P. bogaraveo* from Portuguese waters. Previously, only *A. simplex* s.s., *A. pegreffii*, and *Hysterothylacium* spp. had been detected in this host (Oliveira Rodrigues et al. 1975; Costa et al. 2004; Mladineo 2006). Consequently, *A. simplex* s.s.×*A. pegreffii*, *A. ziphidarum*, *A. typica*, *Anisakis* sp. PB-2009, *Anisakis* sp. PB-2010, *A. physeteris*, and *Contracaecum* sp. are reported for the first time in *P. bogaraveo*. This diversity could be related to the wide habitat range and feeding habits of this host, which feeds on numerous benthic, benthopelagic, and pelagic species, including smaller fish, crustaceans, molluscs, and numerous other

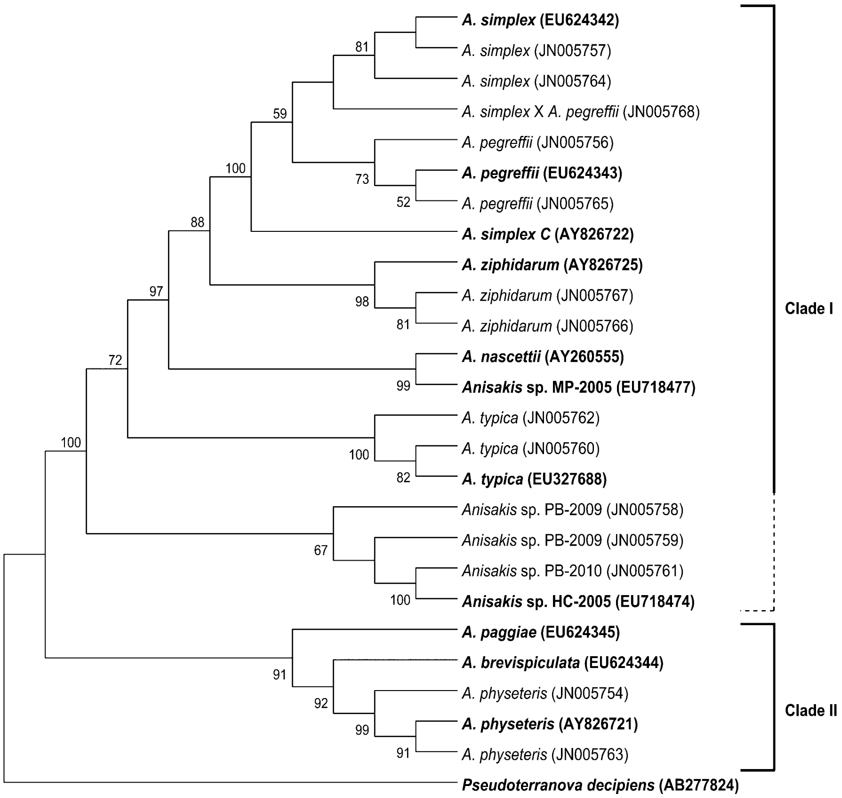

Fig. 3 Neighbour-joining phylogenetic tree of ITS1-5.8S-ITS2 sequences obtained in this study, reference GenBank sequences, and *P. decipiens* as the outgroup. Values beside nodes correspond to bootstrap percentages, and only those above 50% are represented

invertebrates (Morato et al. 2001), thus being exposed to various possible intermediate and paratenic hosts of anisakid nematodes.

Very high prevalences of *Anisakis* spp. infection were detected in mainland Portuguese waters, as reported by other authors (Cruz et al. 2009; Sequeira et al. 2010) and in agreement with findings from Galicia (Abollo et al. 2001; Mattiucci et al. 2004). Characteristic upwelling phenomena occur all along the western Portuguese coast promoting high primary production, feeding a wealth of organisms including the intermediate hosts of anisakid larvae. Anisakid abundance levels were particularly high in Peniche and lower in Sagres, which can be explained by the differences in fish size, since anisakid abundance was found to be positively correlated with fish length. This correlation has been detected in numerous fish species, e.g., *Trachurus trachurus* and *Micromesistius poutassou* (Cruz et al. 2005, 2007) and is rather common for parasite species that have long life spans in the host, since the fish tend to accumulate these parasites as they feed on infected prey.

Almost all of the identified anisakid larvae from mainland waters belonged to the *A. simplex* complex. This coincides with previous studies undertaken in this region (Abollo et al. 2003; Marques et al. 2006; Mattiucci et al. 2008; Sequeira et al. 2010), and it is related to the presence of several suitable definitive hosts, namely, several species of Delphinidae (Santos-Reis and Mathias 1996). However, whereas Mattiucci et al. (2008) found in *T. trachurus* an increasing occurrence of *A. pegreffii* from North to South, and the opposite tendency regarding *A. simplex* s.s., this was not observed in our study in which *A. pegreffii* had a higher relative abundance in all mainland samples.

Hybrid genotypes *A. simplex* s.s.×*A. pegreffii* were detected with relative abundances (9–15%), similar to those found by Abollo et al. (2003) off the mainland Portuguese coast and in the Cantabrian Sea, although lower those found in both the Atlantic and Mediterranean southern coasts of Spain (Abollo et al. 2003; Martín-Sánchez et al. 2005). At the moment, it is not clear whether *A. simplex* s.s. and *A. pegreffii* are in the process of speciation, or if they have been geographically separated in the past, but have secondarily come into contact and, in the absence of barriers, behave as a single species when in sympatry, as suggested by Martín-Sánchez et al. (2005). Furthermore, the occurrence of hybrids in very distant geographical regions, such as China (Du et al. 2010), suggests that this may be a worldwide occurrence whenever *A. simplex* s.s. and *A. pegreffii* occur in sympatry.

Besides *A. simplex* s.l., only three other species were detected in mainland waters: *A. ziphidarum* (Peniche and Sagres), *A. physeteris*, and *Hysterothylacium* sp. (Sagres). *A. ziphidarum* had not been previously detected in the Portuguese mainland waters. This species has beaked whales of the family Ziphiidae as definitive hosts (Mattiucci and Nascetti 2008), and its presence may be explained by the regular occurrence in these waters of Cuvier's beaked whale, *Ziphius cavirostris* (Santos-Reis and Mathias 1996). *A. physeteris* had previously been detected by Sequeira et al. (2010) in Peniche, also in very low numbers, most probably as a result of the rare occurrence of its definitive host *Physeter macrocephalus* (Mattiucci et al. 2001; Santos-Reis and Mathias 1996). *Hysterothylacium* sp. has often been detected in Portuguese waters, both in the mainland (Cruz et al. 2005, 2007) and in Madeira (Costa et al. 2004), where it was also found in the present study.

The diversity of anisakid nematodes isolated from Madeira fish was remarkable (eight species). Previously, both Pontes et al. (2005) and Sequeira et al. (2010) have detected high anisakid species richness (seven species) in fish from Madeira. Studies conducted at similar latitudes (Moroccan and Mauritanian Atlantic coasts) also reported high diversity of anisakid species (Farjallah et al. 2008; Kijewska et al. 2009). The region that includes Madeira, the Canary Islands, and the North African Atlantic coast is located at the confluence of different biogeographic areas, namely the North Atlantic, the Central Atlantic, and the Mediterranean, a factor which greatly contributes to an increased biodiversity. Numerous species of whales and dolphins occur in this region, including the definitive hosts for several anisakid species (Santos-Reis and Mathias 1996; Mattiucci and Nascetti 2008). Madeiran waters seem in fact to represent a region where the distribution of several anisakid species overlaps, as suggested by Pontes et al. (2005), including more typically northern species such as *A. simplex* s.s. and tropical/warmer temperate ones such as *A. typica* (Mattiucci and Nascetti 2008).

The Madeiran anisakid nematode community was dominated by an as yet-undescribed species, *Anisakis* sp. PB-2010. *Anisakis* sp. PB-2010 is most probably the same species as *Anisakis* sp. HC-2005 (Kijewska et al. 2009) and closely related to *Anisakis* sp. PB-2009. This would suggest the existence of several as yet undescribed species in this region, including *Anisakis* sp. B from *Aphanopus carbo* and *Scomber japonicus* (Pontes et al. 2005), *Anisakis* sp. HD, and *Anisakis* sp. type II HD (Sequeira et al. 2010) and *Anisakis* sp. in *Xiphias gladius* from Cape Verde (Garcia et al. 2011).

The Azores sample was markedly different from both Madeira and the mainland. Prevalence and abundance of infection were much lower than in all other samples, including the ones with similar and smaller fish length. Anisakid diversity was also much lower in the Azores than in Madeira, although many marine mammals also occur in this region (Santos-Reis and Mathias 1996). This may be related to different feeding habits of *P. bogaraveo* in this oceanic region. Morato et al. (2001) reported that the main components of the blackspot seabream's diet in Azorean waters are pelagic invertebrates, mainly salps (Thaliaceae), which are not known to harbor any nematode parasites. The dominant species of anisakid in the Azores was *A. physeteris*, with a relative abundance significantly different from all other samples. This was also the dominant species in *Helicolenus dactylopterus* in this region (Sequeira et al. 2010), in sharp contrast to Madeira and the mainland. The occurrence of this parasite in large numbers in the Azores can be due to the presence of a large and stable population of its main definitive host, the sperm whale *P. macrocephalus* (Mattiucci et al. 2001; Pinela et al. 2009).

At the intraspecific level, we found a certain degree of variability, in accordance with findings by other authors (Ceballos-Mendiola et al. 2010; Mattiucci and Nascetti 2007; Palm et al. 2008). Differences were found among individual nematodes both from the same location, as well as from different locations. Indeed, Mattiucci and Nascetti (2008) refer that most of the genetic diversity in *Anisakis* spp. occurs within, rather than among, populations. High intraspecific variability is a feature that can be explained by the environmental heterogeneity experienced by anisakid nematodes with heteroxenous life cycles that involve both ectothermic and endothermic hosts (Mattiucci and Nascetti 2007). At the same time, high rates of gene flow promoted by the high dispersal capacity of their paratenic and especially, definitive hosts, maintain a worldwide homogeneity among populations (Mattiucci and Nascetti 2008).

Conclusions

In conclusion, the diversity of anisakid species in *P. bogaraveo* from Portuguese waters is high. Ten PCR-RFLP patterns were observed, four of which were novel. Seven anisakid taxa were detected in this host for the first time. The phylogenetic analysis (ITS region) revealed that our anisakids are distributed by the two distinct clades reported previously and that a group of organisms, including undescribed specimens from Madeira and a species temporarily described as *Anisakis* sp. HC-2005, cluster together and seem to belong to clade I.

The anisakid nematode communities were relatively similar in all locations in mainland waters but very distinct in both the Azores and Madeira islands, possibly due to differences in the local distribution of both intermediate and definitive hosts. These results suggest the existence of at least three different stocks of *P. bogaraveo* in the northeast Atlantic, but further studies are necessary to confirm this hypothesis.

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Gastrointestinal helminth communities of the blackspot seabream *Pagellus bogaraveo* (Teleostei: Sparidae) from Portuguese north-east Atlantic waters

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Abstract

A study was carried out on the gastrointestinal helminth communities of blackspot seabream, *Pagellus bogaraveo*, from Portuguese mainland and island waters. Fifteen helminth parasite species were detected, including the following new records in this host: the digeneans *Accacladocoelium petasiporum*, *Brachyenteron helicoleni*, *Glomericirrus macrouri*, *Lecithocladium excisum*, *Lepocreadium album*, and *Pachycreadium carnosum*, the nematode *Camallanus* sp. and the acanthocephalan *Rhadinorhynchus pristis*. Infection levels detected were generally low. Samples from mainland waters had a significantly higher prevalence of digeneans than those from the islands, probably due to the greater availability of invertebrate intermediate hosts on the continental slope. The helminth community of *P. bogaraveo* from Azores was diverse but mostly composed of species that infected no more than a single host, whereas in Madeira the helminth community was very depauperate. Most helminth species exhibited niche preferences within the gastrointestinal tract of the host. Higher functional richness and evenness were obtained in helminth communities of *P. bogaraveo* from mainland waters, indicating a balanced use of available resources, which is suggestive of more stable gastrointestinal helminth communities in these waters.

KEYWORDS

Pagellus bogaraveo, blackspot seabream, gastrointestinal parasites, helminths, communities, Atlantic.

Introduction

The blackspot seabream, *Pagellus bogaraveo* (Brünnich, 1768), is a commercially important fish species that occurs in the north-east Atlantic and Mediterranean. It is a benthopelagic fish, occurring at depths of up to 800 m, mostly on the continental shelf break and slope (Spedicato et al. 2002), and on high steepness sites around islands and seamounts in oceanic environments (Menezes et al. 2006). It is an omnivorous fish with a preference for animal prey (Stergiou & Karpouzi 2002), including a wide variety of benthic and pelagic organisms (Morato et al. 2001). Studies of the parasite fauna of this sparid fish are scarce, and most information on its parasites comes from general surveys encompassing several fish species from a certain region. Recently, there have been studies on the anisakid nematodes (Costa et al. 2004a, Hermida et al. 2012) and ectoparasites (Hermida et al. *in press*) of *P. bogaraveo* from Portuguese waters, but the knowledge of its gastrointestinal helminth fauna is very incipient.

The gastrointestinal tract of fish is a rich and complex environment that can be exploited by numerous parasites. The helminth parasite fauna of fish is partly determined by the fish species, due to the varying degrees of phylogenetic specificity exhibited by parasites, but certain features of the host, such as depth range and feeding habits, are also important in influencing the probability of contact with different parasite species, and even more so in the case of gastrointestinal parasites which are trophically acquired. These features can vary geographically within the normal range of the species, due to environmental differences such as topography, resource availability, and local food web structure (Marcogliese 2002).

At the local level, parasite communities are conditioned by the availability of suitable intermediate hosts, which in turn depends on adequate abiotic conditions for their proliferation (Poulin 1997). Local parasite communities can therefore vary widely among the geographical range of the fish host, especially if there is little contact between regions (Poulin 1998). In this study, *P. bogaraveo* specimens from four localities in mainland waters and two Atlantic archipelagos, Madeira and Azores, were sampled and the gastrointestinal communities compared between them.

The ability of different parasite species to exploit specific microhabitats within the host may also affect the structure of parasite communities (Sasal et al. 1999). The distribution of species among available niches and the evenness in resource utilization are important ecological properties of parasite communities that have seldom been studied. Keeney & Poulin (2007) have applied the concepts of functional richness and functional evenness, originally defined in the context of other types of ecological communities, to the study of fish parasite communities, and their results show that functional evenness tends to increase with species richness, which suggests that when new parasite species join a community, they do so by filling empty niches, leading to a more even use of resources when more species are present. The distribution of helminths along the gastrointestinal tract is arguably the most important facet of their niche, because it includes not only their spatial distribution, but also captures other features of their microhabitat such as differences in available nutrients, pH, digestive enzymes of the host, and bacterial flora, among others (Keeney & Poulin 2007).

Materials and methods

Collection and examination of fish

A total of 348 specimens of *P. bogaraveo* from 6 localities in Portuguese waters were obtained from commercial catches between the years of 2009 and 2011. Sampling was carried out during autumn/winter and spring/summer periods, and the sampled localities were the two Atlantic archipelagos of Madeira and Azores and 4 localities along the Portuguese mainland coast, from north to south: Matosinhos, Figueira da Foz, Peniche, and Sagres (Fig. 1). Number of fish analysed from each locality (N) is presented in Table 1. Fish were transported on ice to the laboratory, weighed and measured before being frozen in individual plastic bags. After defrosting, each fish was dissected and analysed for the presence of gastrointestinal helminth parasites. The digestive tract was divided into its natural parts: oesophagus, stomach, pyloric caeca, intestine, and rectum; the intestine was further divided at its natural folds into anterior, mid, and posterior intestine. All parts were separately observed under a stereomicroscope and parasites were collected, counted, cleaned in saline solution, and stored in alcohol 70% (v/v) for further processing. The location of each parasite within the host was recorded. Digenean trematodes and cestodes were stained in carmine following routine laboratory procedures. Nematodes and acanthocephalans were cleared with increasing concentrations of glycerol and temporary mounts were prepared for microscopical observation. All helminths were observed in a Zeiss Axiophot optical microscope and measurements were taken using a digital camera and Axiovision Zeiss Image Analysis System.

Data Analysis

Prevalence and intensity of infection were determined according to Bush *et al.* (1997). Statistical analyses were performed using IBM SPSS statistics software. Whenever possible, prevalence and intensity of infection were compared between all 6 localities studied, and also between the three regions (Mainland, Madeira, and Azores) using a chi-square test for prevalences, and the non-parametric Kruskal-Wallis test for intensities, followed by multiple comparisons whenever significant differences were detected. The correlation between fish size (total length) and parasite species abundance was analysed by Spearman rank correlation. For all tests, statistical significance was accepted when $p<0.05$.

The first-order Jackknife species richness estimator (Jack1) was calculated from abundance data according to Gotelli & Collwell (2011). Simpson's diversity index, evenness index E, and the Berger-Parker dominance index were determined according to Magurran (1988). Functional richness and functional evenness with regard to niche utilization were determined according to Keeney & Poulin (2007), using the E_{var} functional evenness index derived from Smith & Wilson (1996). Functional evenness was determined using abundance data and also using a rough estimate of biomass, which was obtained by multiplying the number of individuals of each species in each niche by their average length (Keeney & Poulin 2007). Whenever possible, length averages were obtained from measurements of a minimum of ten distended individuals. Separate measurements were taken of male and female acanthocephalans, which present sexual dimorphism.

Results

From a total sample of 348 fish obtained from 6 different localities in Portuguese waters, including the two Atlantic archipelagos of Madeira and Azores, fifteen species of gastrointestinal helminths were detected. Digenean trematodes were the most diverse group overall. Ten digenean species were detected: the hemiurids *Lecithocladium excisum* (Rudolphi, 1819), *Hemiurus communis* Odhner, 1905, and *Glomericirrus macrouri* (Gaevskaja, 1973), the derogenid *Derogenes varicus* (Müller, 1784), the accacoelid *Accacladocoelium petasiporum* (Odhner, 1928), the opecoelids *Pycnadenoides senegalensis* Fischthal & Thomas, 1972, and *Pachycreadium carnosum* (Rudolphi, 1819), the lepocreadiid *Lepocreadium album* (Stossich, 1890), and the zoogonids *Diphterostomum vividum* (Nicoll, 1912) and *Brachyenteron helicoleni* Bray & Kuchta, 2006. In addition, some damaged specimens were detected which could only be identified as Hemiuridae gen. sp. and Opecoelidae gen. sp. Other helminths detected were the acanthocephalan *Rhadinorhynchus pristis* (Rudolphi, 1802), the nematode *Camallanus* sp. Railliet & Henry, 1915, and Tetraphyllidea gen. sp. cestode larvae. Local, regional, and overall prevalence values are presented in Table 1, as well as statistically significant differences detected between localities or regions.

A striking difference between mainland Portuguese waters and the islands (Madeira and Azores) is the prevalence of digeneans, which was significantly higher in all mainland samples and much lower in both archipelagos. This same pattern was observed for the hemiurid *L. excisum*, which had a global prevalence of 20.4% in the mainland and was the most prevalent species in Figueira da Foz and Sagres, but did not occur in Madeira and had a very low prevalence in the Azores. The most prevalent species overall was the zoogonid *D. vividum*, which was also the most prevalent species in the two other mainland localities, Matosinhos and Peniche, in the latter having the same prevalence as another zoogonid, *B. helicoleni*, which occurred in all mainland samples but not in the islands. *D. vividum* did not occur in the Azores, and had a lower prevalence in Madeira (8.9%) than in any mainland location; even so, it was the most prevalent species in this region, where the gastrointestinal helminth community was most depauperate. In the Azores, all species detected had extremely low prevalences, the most prevalent species being the acanthocephalan *R. pristis*.

Intensity levels were generally low, and no significant differences were detected between localities or regions (Table 2). The helminth species with highest intensity values were the two zoogonid digeneans, *D. vividum* and *B. helicoleni*, which presented mean intensities of 10.3 ± 27.0 and 49.6 ± 127.3, respectively, and occasionally reached very high numbers in individual hosts (175 *D. vividum* in a fish from Figueira da Foz, and 533 *B. helicoleni* in a fish from Peniche). The only other species to register more than 10 individuals in a single host was *R. pristis*, of which an infrapopulation of 12 individuals (7 males and 5 females) was detected in a fish from Azores.

Positive correlations were found between fish length and abundance of *R. pristis* ($r = 0.111$, $p = 0.039$) and of *P. senegalensis* ($r = 0.139$, $p = 0.010$).

Several ecological parameters were determined in order to compare the six gastrointestinal helminth communities studied (table 3). Observed species richness was higher in Figueira da Foz (10 species) and lower in Madeira, where only 3 helminth species were detected. The helminth community of fish from Azores, although presenting high species richness (8) was mostly composed of species that infected no more than a single host. Species richness estimated by the Jackknife 1 method was highest in Figueira da Foz and Azores. Both diversity and evenness were higher in Matosinhos and Sagres. Madeira had the less diverse and more uneven helminth community. The dominant species was always a digenean of the family Zoogonidae except in the Azores, where the acanthocephalan *R. pristis* was the dominant species. *D. vividum* dominated the communities from Figueira da Foz and Madeira, and *B. helicoleni* was the dominant species in the remaining mainland localities.

Several species exhibited a preference for particular niches within the gastrointestinal tract of the host. The niche range of each species is presented in table 4. Hemiurids were mainly found in the stomach, the lepocreadiid *L. album* in the pyloric caeca, and the zoogonids *B. helicoleni* and *D.*

vividum were mostly detected in the posterior intestine and the rectum, respectively. The acanthocephalan *R. pristis* had the widest range of all species, occurring in all portions of the digestive tract except the rectum.

The parasite communities of fish from Matosinhos, Figueira da Foz and Peniche exhibited the highest functional richness, meaning that all portions of the gastrointestinal tract considered were occupied by at least one parasite species, whereas in Madeira functional richness was very low, with only two subsections of the intestine being utilized by helminths. Functional evenness was highest in Peniche when calculated from abundance data, but it was higher in Madeira when biomass estimates were used instead; in both cases it was lowest in the Azores community.

Discussion

Parasite species and infection levels

The hemiurid *L. excisum* was the most prevalent helminth in *P. bogaraveo* from mainland waters, as well as the second most prevalent species overall, but did not occur in Madeira and had a very low prevalence in the Azores. This parasite was also found to be absent in *Scomber colias* from Madeira and the Canary Islands, whereas it was present in Morocco and Mauritania, and also, albeit with a much lower prevalence, in the Azores (Shukhgalter 2004, Oliva et al. 2008, Costa et al. 2011). It is a euryxenic parasite that occurs in the stomach of numerous fish hosts, and had previously been detected in Portugal (Bray 1973, Rego et al. 1985, Sequeira et al. 2010). The significantly higher prevalence of *L. excisum* in mainland waters may be related to higher availability of benthic invertebrates that serve as intermediate hosts for this species, especially harpacticoid copepods and polychaetes (second intermediate hosts), in the continental shelf and slope when compared with oceanic habitats such as seamounts (Koie 1991, Marcogliese 2002). These invertebrates tend to increase in eutrophic environments (Pérez-del-Olmo et al. 2007), whereas oceanic habitats are characterized by oligotrophic waters and so do not favour their proliferation.

Other hemiurids occurred only in mainland waters (*H. communis*, *G. macrouri,* and Hemiuridae gen. sp.), but with low prevalences. *Hemiurus communis* is a euryxenic digenean which also uses harpacticoid copepods as second intermediate hosts (Koie 1995), and had been previously detected in *P. bogaraveo* from the Atlantic (Nicoll 1914, Little 1929), whereas *G. macrouri* is a parasite of macrourids that has also been detected in other deep-water fishes (e.g. *Hoplostethus atlanticus*) at depths of 500-2000m, occurring on the edge of the continental shelf on both sides of the Atlantic (Gibson & Bray 1986). The occurrence of a single individual of this species in a *P. bogaraveo* specimen from Figueira da Foz is likely to be accidental and due to a similarity of habitat and feeding habits.

The derogenid *D. varicus*, another euryxenic species, is one of the most widely distributed digeneans worldwide, usually occurring in small numbers (Manter 1954, Koie 1979), and had previously been detected in *P. bogaraveo* from the north-east Atlantic (Little 1929). Two of the three specimens detected were found in the gill chamber of the host, but this might result from a post-mortem migration from the stomach, as also observed by Kostadinova et al. (2003), which is likely since they tend to occupy the cardiac end of the stomach and sometimes the oesophagus (Blair et al. 1998).

Accacladocoelium petasiporum infected a single fish from Azores. This species had been previously detected in this region in the sunfish *Mola mola*, at a depth of 245 m (Gaevskaya 2002). Since sunfish are known to feed mostly on gelatinous zooplankton (Pope et al. 2010), these are expected to be the main intermediate hosts of accacoeliid trematodes (Bray & Gibson 1977). *P. bogaraveo* is an omnivorous species that feeds on both benthic and pelagic prey (Morato et al. 2001), and may therefore have acquired this infection accidentally through the ingestion of infected gelatinous organisms.

The opecoelid *P. senegalensis* has been reported from *P. bogaraveo* in the Atlantic and Mediterranean (see references in Bartoli et al. 1989). Both this species and *P. carnosum*, which was detected here in *P. bogaraveo* for the first time, seem to show stenoxenic specificity towards the Sparidae (Bartoli et al. 2005). This is also the case for *L. album*, which has been reported mostly from sparid fish in the north-east Atlantic and Mediterranean (Bartoli et al. 2005).

The most prevalent species overall was the zoogonid *D. vividum*, which is the only species detected in this study that is strictly oioxenic towards its definitive host, *P. bogaraveo* (Bray 1987), having been reported from the north-east Atlantic (Nicoll 1912, 1914, Bray & Gibson 1986). The other zoogonid detected, *B. helicoleni*, had only previously been recorded in small numbers from *Helicolenus dactylopterus* off Scotland, at a depth of 1600 m (Bray & Kutcha 2006). Its presence in *P. bogaraveo* seems to confirm a preference for the deep-water environment. In the present study, *B. helicolenus* occurred in all mainland localities but not in the islands. Interestingly, the highest prevalence was detected in the sample from Peniche, whereas a recent parasitological study of *H. dactylopterus* from the same locality failed to yield any specimens of this zoogonid (Sequeira et al. 2010), suggesting that *H. dactylopterus* is not its main host in Portuguese waters. However, at the moment it seems premature to try to assess its specificity.

Rhadinorhynchus pristis was the most prevalent species in Azores, having also occurred in all mainland samples, but not in Madeira, which is unexpected since it has been previously detected in other fish species from that region by Costa et al. (2004b, 2009). Like most acanthocephalans, it shows little specificity, and its hosts are more likely to be related ecologically through similar diets and habitat preferences that phylogenetically (Kennedy 2006). The higher prevalence of *R. pristis* in Azores might reflect a greater amount of pelagic crustaceans in the diet of *P. bogaraveo* from this region, as these are used as intermediate hosts by this acanthocephalan (Klimpel et al. 2006).

Helminth Communities

Two main patterns were evident in this study: on the one hand, the mainland helminth communities of *P. bogaraveo* exhibited significantly higher infection levels, especially of digeneans, when compared to the islands, and on the other hand, the two archipelagos differed a great deal in terms of diversity, with Azores showing higher diversity and evenness, whereas Madeira had a very impoverished helminth community. Sasal *et al.* (1999) found the diet of the host to be the most important factor affecting digenean diversity in sparid fish from the Mediterranean. *P. bogaraveo* is a benthopelagic fish with a diverse omnivorous diet that includes benthic and pelagic organisms (Morato et al. 2001), and can thus be in contact with a wide range of potential intermediate and paratenic hosts of helminth parasites. However, helminth richness will be locally conditioned by the availability of suitable intermediate hosts, especially benthic invertebrates in the case of digeneans.

The high diversity and infection levels of digeneans in mainland waters may reflect the high diversity of benthic fauna in continental slope waters, as many of these organisms can serve as intermediate hosts for trematodes (Bray et al. 1999, Marcogliese 2002). Within mainland waters, the sample from Peniche had the lowest diversity and evenness, as well as the highest prevalence of both zoogonid species. These differences might be related to underwater topology, since one of the largest submarine canyons in Europe occurs in this region, promoting organic enrichment due to high levels of sedimentation and thus causing a severe reduction in diversity of benthic organisms (Cúrdia et al. 2004).

Both island systems presented low helminth infection levels, which may be related to the low availability of suitable intermediate hosts. Oceanic environments do not present favourable conditions for the presence of many benthic invertebrates and, even when such conditions are present, such as in seamounts, many invertebrate species are limited in their distributions due to low dispersal capacity (Parker & Tunnicliffe 1994). Although in Azores helminth diversity was relatively high, all digenean species were rare, occurring in only one fish specimen, and may represent either accidental infections, or reflect migratory events.

The high values of estimated species richness in Figueira da Foz and Azores reflect the high proportion of rare species found in these localities, since the Jackknife 1, like other non-parametric species richness estimators, uses the proportion of rare species detected in the community to infer the existence of undetected species (Gotelli & Colwell 2011). This is one of the recommended methods to control for the effect of differences in sampling effort on estimates of total parasite species richness (Walther & Morand 1998). It provides a good estimation of the minimum number of species existent in a community (Gotelli & Colwell 2011). However, care must be taken in interpreting these results, since many of the rare species detected are probably accidental and their biological significance to the helminth communities of *P. bogaraveo* is therefore limited. Nonetheless, they reflect the wide diet range of this fish in this region (Morato et al. 2001), which seems to promote contact with many parasite species.

The gastrointestinal helminth community of *P. bogaraveo* from Madeira was the less rich, and presented the lowest diversity and evenness. Costa et al. (*in press*) also detected a less rich helminth parasite fauna in *Trachurus picturatus* from Madeira when compared with Azores and with a mainland sample from the West African Coast. Both in the case of *T. picturatus* (Costa et al. *in press*) and in the present study, this low diversity reflects a depauperate digenean fauna, which probably results from the absence of suitable intermediate hosts for many species. Furthermore, benthic production is known to decrease at lower latitudes and a shift to pelagic prey tends to occur in subtropical and tropical waters (Marcogliese 2002).

The two zoogonid species exhibited the highest abundance, and dominated all but one of the 6 component communities in this study. This seems to be in line with the tendency for small-bodied species to occur at higher densities than larger species in most ecological communities, including in parasite communities (Poulin et al. 2008), but it might also reflect, in the case of *D. vividum*, the strict specificity of this species (Bray 1987). Specialization might provide an advantage in competition with euryxenic species, as there is often a trade-off between the ability to use many host species and the average fitness achieved in these hosts (Poulin 1998).

Azores was the only locality where the dominant species was an acanthocephalan. Helminth communities of marine fish are most often dominated by digeneans, and the occurrence of acanthocephalans is related to host diet (Kennedy 2006). The dominance of the *P. bogaraveo* helminth community by the acanthocephalan *R. pristis* in Azores has to be viewed in the light of the low abundance of digeneans in this region.

Niche ranges and resource utilization

Microhabitat distribution within the gastrointestinal tract has been shown to be an important factor in determining the structure of helminth parasite communities (Sasal et al. 1999). In this study several species presented a clear preference for certain regions of the gastrointestinal tract. Unsurprisingly, almost all hemiurids were found in the stomach. With a thick tegument and retractile ecsoma, these digeneans seem to be well adapted to the harsh stomach environment (Gibson & Bray 1986), which is often avoided by other helminths due to the low pH.

The zoogonid *D. vividum*, on the other hand, exhibits a preference for the rectum (Bray & Gibson 1986), and in this study 99.7% of all specimens of this zoogonid were in fact detected in that particular niche. The other zoogonid detected, *B. helicoleni*, had a slightly wider niche range, occurring throughout the intestine, but with 92% of specimens occurring in the posterior intestine, prior to the rectum. The different niche preferences on the part of the two zoogonids may be due to specific requirements, e.g. in terms of nutrients, or it may be related to competition between them, either past or present. Intense competition between abundant species may favour evolutionary divergence of their fundamental niches, leaving only the so-called 'ghost of competition past' (Poulin 2001), and thus effectively avoiding competition. Interestingly, although the two species co-occured in 9 fish, they never shared the same microhabitat, and in two cases *D. vividum* individuals occurred in the mid-intestine, but not in the posterior intestine, when there were *B. helicoleni* specimens in that niche, suggesting that *D. vividum* individuals might be occasionally displaced by the other zoogonid, and that current competition between these species may be important in defining their respective niches.

The opecoelid *P. senegalensis* occurred mostly in the anterior and mid-intestine, and *L. album* was mostly (88%) detected in the pyloric caeca, as was also verified by Sasal et al. (1999) in several sparid hosts. Other species occurred in very low numbers, precluding any meaningful observations on their niche range in this host.

The acanthocephalan *R. pristis* occurred in all regions except the rectum. The wide niche range of this species within the host's gastrointestinal tract might be related to its low specificity, since the anatomy of fish digestive tracts is very variable, which might prevent the evolution of niche specialization in parasites with low specificity towards the fish host. In addition, many acanthocephalan species are known to move within the digestive tract of the host, thus increasing their mating opportunities (Crompton 1970).

The distribution of organisms in niche space is one of the factors that can determine community properties. Recently, Keeney & Poulin (2007) have applied the concepts of functional richness and functional evenness to fish parasite communities. Functional richness is a measure of the proportion of gastrointestinal subsections that are used by at least one worm, whereas functional evenness measures the evenness of distribution of helminths across all occupied niche sections (Keeney & Poulin 2007). In the present study, functional evenness was generally higher than in the cestode communities of elasmobranch hosts analysed by Keeney & Poulin (2007), and functional richness was similar (between 0.83 and 1.00), except in Madeira where the very impoverished helminth community occupied only 2 of 6 niches within the gastrointestinal tract of the host. Unlike the results of those authors, however, in the present study functional evenness values based on biomass estimates were actually lower than those based on abundance data, except for the Azores community. In the light of the extreme size differences between helminth species, even a rough estimate of biomass such as the one used here is a major improvement over abundance data per se (Keeney & Poulin 2007). Functional evenness calculated using estimated biomass is likely to be a more realistic measure of resource utilization by the helminth community.

Nonetheless, functional evenness must be understood in conjunction with functional richness. Thus, while the Madeira community has a high functional evenness, this refers to the utilization of only two intestinal regions on the part of a very impoverished helminth community, which can be seen by the low functional richness value, indicating that resources are in fact underutilized. In contrast, helminth communities from mainland waters, with a high functional richness and evenness, seem to demonstrate a balanced utilization of niche space, and are likely to represent more stable gastrointestinal helminth communities.

The Azores community clearly shows the lowest functional evenness, whether abundance or biomass estimates are used. This low functional evenness reflects the uneven resource use on the part of a helminth community which, although presenting high species richness, diversity, and evenness, is in fact constituted almost entirely of rare species. In these circumstances, functional richness and evenness seem to capture relevant aspects of community structure which other commonly used ecological indices miss.

In conclusion, gastrointestinal helminth communities of *P. bogaraveo* from mainland waters not only presented higher diversity and infection levels, especially of digenean species, but were characterized by high functional richness and evenness, indicating stable communities. In contrast, the gastrointestinal helminth community from Madeira was very depauperate and presented low functional richness, whereas the Azores community, although diverse, had very low functional evenness. These differences are likely to reflect biogeographical differences in the availability of benthic invertebrates and a shift to a more pelagic diet in subtropical waters.

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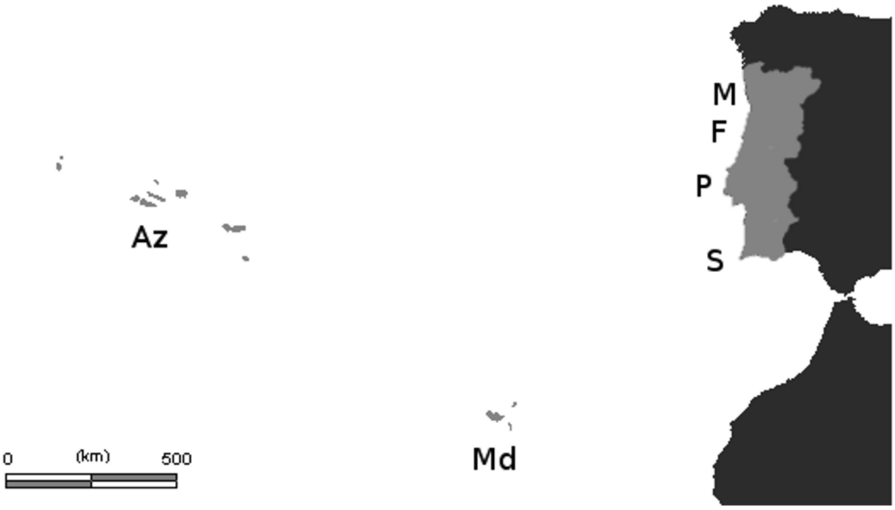

Fig. 1. Sampling localities in the Portuguese mainland coast (M, Matosinhos; F, Figueira da Foz; P, Peniche; S, Sagres) and islands (Md, Madeira; Az, Azores).

Table 1. Prevalence (%) of gastrointestinal helminth infection in *Pagellus bogaraveo* from Mainland Portugal (Matosinhos, Figueira da Foz, Peniche, and Sagres) and Atlantic archipelagos (Madeira, Azores). Number (N) and total length (L) (mean ± standard deviation, in cm) of fish examined. Significant differences detected among 6 localities and among 3 regions (Mainland, Madeira, and Azores) by chi-square test. Similar letters indicate no statistically significant differences between localities and/or regions.

Parasite Species	Specificity	Matosinhos N = 42 L = 23.0 ± 2.6	Figueira da Foz N = 92 33.0 ± 1.8	Peniche N = 42 37.4 ± 3.1	Sagres N = 30 30.0 ± 0.7	Madeira N = 56 31.0 ± 2-6	Azores N = 86 34.9 ± 4.7	Total Mainland N = 206 33.2 ± 3.1	Overall N = 348 33.3 ± 3.7	Significant differences
Lecithocladium excisum	E	9.5	29.3	7.1	26.7	0.0	1.2	20.4	12.4	
		ab	c	bd	ac	e	de			6 loc: p=0.000
						a	a	b		3 reg: p=0.000
Hemiurus communis	E	4.8	1.1	0.0	0.0	0.0	0.0	1.5	0.9	
Glomericirrus macrouri	A	0.0	1.1	0.0	0.0	0.0	0.0	0.5	0.3	
Hemiuridae gen. sp.	U	0.0	1.1	0.0	3.3	0.0	0.0	1.0	0.6	
Derogenes varicus	E	0.0	2.2	0.0	0.0	0.0	1.2	1.0	0.9	
Accacladocoelium petasiporum	A	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.3	
Pachycreadium carnosum	S	0.0	0.0	0.0	0.0	1.8	1.2	0.0	0.6	
Pycnadenoides senegalensis	S	2.4	3.3	11.9	0.0	0.0	1.2	4.4	2.9	
Opecoelidae gen. sp.	U	2.4	0.0	0.0	0.0	0.0	0.0	0.5	0.3	
Lepocreadium album	S	7.1	10.9	2.4	0.0	0.0	1.2	6.8	4.3	
Brachyenteron helicoleni	U	7.1	1.1	23.8	10.0	0.0	0.0	8.3	4.9	
Diphterostomum vividum	O	14.3	17.4	23.8	23.3	8.9	0.0	18.9	12.6	
		ab	ab	b	ab	a	c			6 loc: p=0.000
						a	b	a		3 reg: p=0.000
TOTAL DIGENEA	-	33.3	48.9	45.2	43.3	8.9	5.8	44.2	29.0	
		a	a	a	a	b	b			6 loc: p=0.000
						b	b	a		3 reg: p=0.000
Tetraphyllidea gen. sp.	E	0.0	0.0	2.4	0.0	1.8	1.2	0.5	0.9	
Camallanus sp.	U	0.0	0.0	2.4	0.0	0.0	0.0	0.5	0.3	
Rhadinorhynchus pristis	E	2.4	1.1	4.8	3.3	0.0	7.0	2.4	3.2	

O, oioxenic (strictly specific to *P. bogaraveo*); S, stenoxenic for Sparidae; E, euryxenic; U, specificity unknown; A, accidental occurrence; p, probability level.

Table 2. Intensity (mean ± sd, min-max) of gastrointestinal helminths in *P. bogaraveo* from Mainland Portugal (Matosinhos, Figueira da Foz, Peniche, and Sagres) and Atlantic archipelagos (Madeira and Azores).

Parasite Species	Matosinhos	Fig. Foz	Peniche	Sagres	Madeira	Azores	Mainland	Overall
Lecithocladium excisum	2.5±3.0 (1-7)	2.3±1.7 (1-6)	1.3±0.6 (1-2)	1.8±1.8 (1-6)	-	1.0 (1)	2.1±1.8 (1-7)	2.1±1.8 (1-7)
Hemiurus communis	1.0±0.0 (1)	1.0 (1)	-	-	-	-	1.0±0.0 (1)	1.0±0.0 (1)
Glomericirrus macrouri	-	1.0 (1)	-	-	-	-	1.0 (1)	1.0 (1)
Hemiuridae gen. sp.	-	1.0 (1)	-	1.0 (1)	-	-	1.0±0.0 (1)	1.0±0.0 (1)
Derogenes varicus	-	1.0±0.0 (1)	-	-	-	1.0 (1)	1.0±0.0 (1)	1.0±0.0 (1)
Accacladocoelium petasiporum	-	-	-	-	-	3.0 (3)	-	3.0 (3)
Pachycreadium carnosum	-	-	-	-	1.0 (1)	1.0 (1)	-	1.0±0.0 (1)
Pycnadenoides senegalensis	1.0 (1)	2.3±2.3 (1-5)	3.0±3.6 (1-9)	-	-	1.0 (1)	2.5±3.0 (1-9)	2.3±2.8 (1-9)
Opecoelidae gen. sp.	1.0 (1)	-	-	-	-	-	1.0 (1)	1.0 (1)
Lepocreadium album	1.0±0.0 (1)	1.9±1.4 (1-5)	1.0 (1)	-	-	1.0 (1)	1.6±1.2 (1-5)	1.6±1.2 (1-5)
Brachyenteron helicoleni	30.7±49.7 (2-88)	2.0 (2)	70.4±164.2 (1-533)	15.0±12.5 (3-28)	-	-	49.6±127.3 (1-533)	49.6±127.3 (1-533)
Diphterostomum vividum	9.8±13.5 (1-31)	14.5±43.0 (1-175)	8.4±8.6 (1-26)	2.0±1.2 (1-4)	13.0±17.5 (1-43)	-	10.0±28.1 (1-175)	10.3±27.0 (1-175)
TOTAL DIGENEA	12.0±23.9 (1-88)	7.3±26.1 (1-177)	42.4±126.8 (1-559)	5.7±8.1 (1-29)	13.2±17.5 (1-43)	1.6±0.9 (1-3)	15.1±62.0 (1-559)	14.4±59.0 (1-559)
Tetraphyllidea gen. sp.	-	-	1.0 (1)	-	1.0 (1)	1.0 (1)	1.0 (1)	1.0±0.0 (1)
Camallanus sp.	-	-	1.0 (1)	-	-	-	1.0 (1)	1.0 (1)
Rhadinorhynchus pristis	1.0 (1)	1.0 (1)	1.0±0.0 (1)	2.0 (2)	-	4.0±4.8 (1-12)	1.2±0.4 (1-2)	2.7±3.7 (1-12)

Table 3. Ecological parameters of the gastrointestinal helminth communities of *Pagellus bogaraveo* from different localities in Portuguese waters.

	Matosinhos	Figueira da Foz	Peniche	Sagres	Madeira	Azores
N	42	92	42	30	56	86
Observed species richness	8	10	8	5	3	8
Estimated sp richness (Jack1)	11	14	11	6	5	14
Simpson's diversity index	2.368	1.852	1.305	2.384	1.062	1.843
Evenness index E	0.660	0.511	0.267	0.726	0.088	0.523
Berger-Parker dominance index	0.544	0.707	0.869	0.592	0.970	0.727
Dominant species	*B. helicoleni*	*D. vividum*	*B. helicoleni*	*B. helicoleni*	*D. vividum*	*R. pristis*
Functional richness	1.00	1.00	1.00	0.83	0.33	0.83
Functional evenness A	0.747	0.764	0.821	0.750	0.797	0.270
Functional evenness B	0.641	0.746	0.669	0.729	0.756	0.428

A, based on abundance data; B, based on estimated biomass.

Table 4. Niche range of gastrointestinal helminth parasites of *P. bogaraveo* and number of helminths of each species detected in each subsection. Five shades of grey indicate, from light to darker, 1, 2-9, 10-49, 50-99, and ≥ 100 individuals, respectively.

	Stomach	Pyloric caeca	Anterior intestine	Mid-intestine	Posterior intestine	Rectum
Lecithocladium excisum	90		1			
Hemiurus communis	3					
Glomericirrus macrouri	1					
Hemiuridae gen. sp.	1				1	
Derogenes varicus	1*					
Accacladocoelium petasiporum	2				1	
Pachycreadium carnosum		2				
Pycnadenoides senegalensis		1	12	9		
Opecoelidae gen. sp.					1	
Lepocreadium album	2	21	1			
Brachyenteron helicoleni			10	56	772	5
Diphterostomum vividum			1	2		451
Tetraphyllidea gen. sp.		1		1	1	
Camallanus sp.		1				
Rhadinorhynchus pristis	1	9	12	7	1	

*Two additional specimens were detected on the gills, but were considered to have migrated to that location, and hence were disregarded in this analysis.

CHAPTER 4

Parasites of *P. bogaraveo* as Biological Tags

SELECTION OF PARASITE TAGS FOR BLACKSPOT SEABREAM

At the beginning of this study there was very little information on the parasite fauna of blackspot seabream, and even less so in the north-east Atlantic, therefore a thorough survey of the parasites of this fish in the study area was required, as recommended by MacKenzie & Abaunza (2005). After information was gathered on the occurrence and infection levels of parasites of *P. bogaraveo* in the study area, in the fifth paper (Hermida M., Cruz C., Saraiva A. Parasites as biological tags for stock identification of blackspot seabream, *Pagellus bogaraveo*, in north-east Atlantic waters, s*ubmitted for publication*) parasite species were selected as tags for the study of blackspot seabream stocks in north-east Atlantic waters, according to the criteria outlined in Lester (1990), MacKenzie & Abaunza (1998, 2005), and Lester & MacKenzie (2009).

Parasite species with significantly different prevalence values between localities were pre-selected as potential biological tags. Within these species, only those with a relatively long permanence time in the host were considered appropriate, and no species known to have serious pathogenic effects were used. The following parasite species were selected as biological tags of *P. bogaraveo* in the north-east Atlantic: *Diphterostomum vividum* (Digenea: Zoogonidae), *Anisakis simplex* s.l., *A. physeteris*, *Anisakis* sp. PB-2010 (Nematoda: Anisakidae), and *Bolbosoma* sp. (Acanthocephala: Polymorphidae). All these, except for the digenean, occured in *P. bogaraveo* as juveniles, generally encysted in the body cavity and mesenteries, or attached to the outer part of visceral organs.

BLACKSPOT SEABREAM STOCKS IN THE NORTH-EAST ATLANTIC

Significant differences in the infection levels of the species mentioned above clearly indicated a separation between samples from three regions: the mainland, Madeira, and the Azores. Mainland samples were clearly identified by the significantly higher relative abundance of *A. simplex* s.l. and the significantly lower prevalence of *Bolbosoma* sp. The blackspot seabream population from Madeira was characterized by the high relative abundance of an anisakid species that occurred nowhere else, *Anisakis* sp. PB-2010, and significantly lower relative abundance of species from the *A. simplex* s.l. complex when compared to both mainland and Azorean waters. The sample from the Azores was mainly characterized by the significantly higher relative abundance of *A. physeteris*, absence of the digenean *D. vividum*, and by overall low anisakid infection levels.

These results point to the existence of three separate stocks of *P. bogaraveo* in the study area: one in mainland Portugal, one in Madeira and another in the Azores, findings which are further corroborated by the very high prevalence of the copepod *Hatschekia pagellibogneravei* in Madeira and its absence from mainland waters, which seems to indicate a lack of migration of blackspot seabream, at least from Madeira to the mainland. Although *H. pagellibogneravei* was found to be inadequate as a tag for the purpose of stock identification, it can nevertheless be useful for the study of fish migrations (Lester 1990, Moore 2001).

The separation of the Azorean blackspot seabream population is partly supported by genetic studies; however, no genetic differentiation has so far been detected between mainland and Madeiran populations. Nevertheless, that should not be taken to imply panmixia, especially since blackspot seabream evidence very little genetic variability, consistent with an historical bottleneck (Stockley et al. 2005).

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Parasites as biological tags for stock identification of blackspot seabream, *Pagellus bogaraveo* in north-east Atlantic waters

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Abstract

In this study parasites were selected as biological tags for the identification of blackspot seabream (*Pagellus bogaraveo*) stocks in the north-east Atlantic: *Diphterostomum vividum* (Digenea: Zoogonidae), *Anisakis simplex* s.l., *A. physeteris*, *Anisakis* sp. PB-2010 (Nematoda: Anisakidae), and *Bolbosoma* sp. (Acanthocephala: Polymorphidae). Those parasite species point to the existence of three distinct blackspot seabream stocks in the north-east Atlantic: one in the Azores region (ICES Area X), one in continental Portuguese shelf/slope waters (ICES Area IXa), and a third in the waters around Madeira (sub-area 1.2 of FAO 34, central-eastern Atlantic).

Keywords

Atlantic, biological tags, parasites, blackspot seabream, *Pagellus bogaraveo*, populations, stocks.

Introduction

The blackspot seabream, *Pagellus bogaraveo* (Brünnich, 1768) is a benthopelagic sparid fish that occurs in the north-east Atlantic and Mediterranean Sea, mostly on the continental shelf break and slope, on seamounts and around islands, at depths of up to 800 m (Spedicato et al. 2002, Menezes et al. 2006). It is a slow growing and relatively long-lived species, with a protandric hermaphroditic reproductive system, all features that render it particularly vulnerable to fishing pressure, which indeed has already caused the collapse of the Cantabrian Sea stock (Lorance et al. 2011). At present, the most important grounds for commercial fisheries of blackspot seabream are the waters around the Azores archipelago (ICES Area X).

Very little is known about the population structure of this important sparid fish in the north-east Atlantic. Studies of mitochondrial DNA markers show that *P. bogaraveo* exhibits an exceptionally low genetic variability, consistent with the occurrence of a past bottleneck (Bargelloni et al. 2003, Stockley et al. 2005, Lemos et al. 2006), which has been estimated to have happened in the late Pleistocene, a period characterized by strong climatic change (Patarnello et al. 2007). Stockley et al. (2005), however, point out that recent effects of fishing, especially the reduction in the number of females, could also have contributed to the low diversity in the mitochondrial genome, by drastically reducing maternal population size.

A study using microsatellite markers found no significant genetic differentiation between Mediterranean and Atlantic blackspot seabream populations along the European continental shelf/slope, which might be due to the absence of strong barriers to gene flow (Piñera et al. 2007), whereas a morphological study detected some variation in morphometric features between populations, but no geographical gradient to this variation (Palma & Andrade 2004). Within the north-east Atlantic significant, albeit low, genetic variation has been detected in the mitochondrial D-loop region and microsatellite markers between populations from mainland Portuguese waters and the Azores (Stockley et al. 2005, Lemos et al. 2006).

Identifying stocks is of primary interest to fisheries management because population dynamics such as recruitment and the response to fishing pressure can operate independently on individual stocks (Waldman, 1999). Even though at first sight there appear to be less conspicuous barriers in the ocean than in other ecosystems, marine fish can exhibit a complex population structure, with varying degrees of reproductive isolation (Abaunza et al. 2008). However, in some cases, such as when recent recolonization has occurred after an evolutionary bottleneck event, population structure may be effectively invisible to the most commonly used genetic markers; in such cases, using an additional non-genetic approach, such as biological tagging, can enhance resolution (Waldman 1999).

Parasitological approaches can be excellent indicators of stock separation (Pawson & Jennings 1996). Parasites can be used as biological tags since their geographic ranges are limited by the abiotic and ecological conditions that permit their development and completion of their life cycles. The principle of the method is that a fish can only become infected with a parasite when entering its endemic range (MacKenzie & Abaunza 2005). In order to qualify as an appropriate biological tag, a parasite must be present with different infection levels in the fish host within the study area, must remain in the host for long periods of time (usually more than a year), and should not cause serious pathology or alter its host's behaviour (MacKenzie & Abaunza 1998, 2005).

Due to the almost complete lack of information on the parasite fauna of blackspot seabream in the north-east Atlantic Ocean, a comprehensive survey was carried out in the study area, in order to permit selection of appropriate parasite tags, as recommended by MacKenzie & Abaunza (2005). The results of this survey indicated that there were significant differences in occurrence and parasite infection levels in fish from different localities within the study area (Hermida et al. 2012, Hermida et al. *in press*, unpublished results). Here we select appropriate parasite tags for the study of *P. bogaraveo* populations, following the criteria outlined in Lester (1990), MacKenzie & Abaunza (1998, 2005), and further clarified by Lester & MacKenzie (2009). We then proceed to analyse that information with a view to identifying *P. bogaraveo* stocks in the study area. The main aim of this work is to provide additional information that will complement genetic studies, in order to achieve a better understanding of the population structure of blackspot seabream in north-eastern Atlantic waters.

Material and Methods

A comprehensive study on the metazoan parasite fauna of blackspot seabream in the north-east Atlantic was carried out from 2009 to 2011. A total of 348 fish were obtained from commercial catches. Sampling locations included the archipelagos of Madeira (56) and Azores (86), and four localities in the Portuguese mainland coast: Matosinhos (42), Figueira da Foz (92), Peniche (42), Sagres (30).

Fish were dissected and observed for the presence of metazoan parasites according to routine parasitological techniques. All parasites detected were collected, counted, preserved in 70% (v/v) ethanol and processed according to taxonomic group. Parasites were observed in a Zeiss Axiophot optical microscope and measurements were taken using a digital camera and Axiovision Zeiss Image Analysis System. All parasites except anisakid nematodes were morphologically identified to the lowest possible taxonomic rank following taxonomic guides and original descriptions in the literature. Anisakid nematodes are parasitic in fish as juveniles, and can only be identified to species level using molecular techniques. Due to the large numbers detected, a sub-sample of more than 30 specimens was randomly selected within all the anisakids collected from each locality for identification using PCR-RFLP and/or sequencing of the ITS region (Hermida et al. 2012).

Prevalence of infection was determined according to Bush et al. (1997). Statistical analyses were carried out using IBM SPSS statistics software. Whenever possible, prevalence was compared among all localities and also between the three regions (Azores, Madeira, and the mainland) using a Chi-square test, followed by multiple comparisons whenever significant differences were detected. Statistical significance was accepted when $p<0.05$.

Results

A total of thirty-six parasite species were detected in *Pagellus bogaraveo* from Portuguese waters, including nine ectoparasite species (the monogeneans *Choricotyle chrysophryi* and *Lamellodiscus virgula*, the copepods *Hatschekia pagellibogneravei* and *Peniculus fistula*, the isopods *Aega antillensis*, *A. deshaysiana*, *Gnathia* sp., and *Rocinela danmoniensis*, and the branchiuran *Argulus* sp.) (Hermida et al. *in press*) and the following endoparasites: the myxosporean *Ceratomyxa* sp., the digeneans *Accacladocoelium petasiporum*, *Brachyenteron helicoleni*, *Derogenes varicus*, *Diphterostomum vividum*, *Glomericirrus macrouri*, *Hemiurus communis*, Hemiuridae gen. sp., *Lecithocladium excisum*, *Lepocreadium album*, Opecoelidae gen. sp., *Pachycreadium carnosum*, and *Pycnadenoides senegalensis*, cestode tetraphyllidean larvae, anisakid nematode larvae, the nematodes *Camallanus* sp. and *Philometra filiformis*, and the acanthocephalans *Bolbosoma* sp. (juveniles) and *Rhadinorhynchus pristis*. A subsample of anisakids was molecularly identified to species level, revealing the occurrence of nine anisakid nematode species: *Anisakis simplex* sensu stricto, *A. pegreffii*, *A. ziphidarum*, *A. typica*, *Anisakis* sp. PB-2009, *Anisakis* sp. PB-2010, *A. physeteris*, *Hysterothylacium* sp. PB-2010, and *Contracaecum* sp. PB-2010 (Hermida et al. 2012). Additionally, *A. simplex* s.s. x *A. pegreffii* hybrid genotypes were also detected.

Prevalence values detected in each of the four mainland localities and the two Atlantic archipelagos of Azores and Madeira, as well as significant differences, are presented in table 1. The highest prevalences were obtained for anisakid nematodes in all the studied localities, and also for the copepod *Hatschekia pagellibogneravei* in *P. bogaraveo* from Madeira.

There were significant differences between mainland localities in the prevalence of the digenean *Lecithocladium excisum*, but no clear gradient or distribution pattern could be discerned. Anisakid prevalence also varied significantly between mainland localities, with samples from Matosinhos and Peniche exhibiting the highest prevalence and the sample from Sagres, the lowest. Again there was no clear gradient or pattern to this variation. Although no prevalence values were obtained for different anisakid nematode species, significant differences were detected in the relative abundance of *Anisakis simplex* s.s. and *A. pegreffii* between mainland localities (Hermida et al. 2012). Furthermore, the species *Anisakis ziphidarum*, *A. physeteris*, and *Hysterothylacium* sp. PB-2010 presented differences in occurrence within mainland waters – *A. ziphidarum* occurred in Peniche and Sagres, whereas the latter two occurred only in Sagres – but with very low relative abundance. Relative abundance of *A. simplex* s.l. (which included *A. simplex* s.s., *A. pegreffii*, and hybrids thereof) was very high in all mainland localities, exhibiting no significant differences within this region (Hermida et al. 2012).

When comparing the three regions (mainland Portugal, Madeira and the Azores), significant differences were detected in the prevalence of several parasite species. Among ectoparasites, the diplectanid monogenean *Lamellodiscus virgula* presented a significantly higher prevalence in the mainland when compared to the Azores, although not significantly different from Madeira. The other detected monogenean, *Choricotyle chrysophryi*, occurred only in mainland waters, but prevalence values were too low to allow statistical analysis. The copepod *Hatschekia pagellibogneravei* occurred only in the Atlantic archipelagos, with significantly higher prevalence in Madeira. Another crustacean, *Gnathia* sp., had a significantly lower prevalence in the mainland when compared to both archipelagos and was, in fact, absent from all but one of the mainland localities.

Among endoparasites, significant differences were detected in the prevalence of the digeneans *D. vividum* and *L. excisum* when comparing the three regions. The zoogonid *D. vividum* occurred in all localities except in the Azores, whereas the hemiurid *L. excisum* occurred in all localities except in Madeira, but prevalence was lower in the Azores than in mainland localities. Furthermore, another zoogonid, *B. helicoleni*, occurred in all mainland localities and not in the archipelagos; however the prevalence values detected did not permit statistical analysis. The *A. simplex* s.l. complex also exhibited significant differences between the three regions, with relative abundance being very high in all mainland localities, lower in the Azores, and much lower in Madeira. Two other anisakid species also presented clear and significant regional differences: *A. physeteris* had a significantly higher relative abundance in the Azores, and *Anisakis* sp. PB-2010 occurred only in blackspot seabream from Madeira, with a statistically significant high relative abundance (Hermida et al. 2012).

Discussion

Selection of parasite tags

Lester (1990) considered the life span to be the single most important criterion for the suitability of a parasite as a biological tag. Following this recommendation, it is evident that the most adequate tags from the parasites detected are those that occur as juveniles, namely anisakid nematodes and the acanthocephalan *Bolbosoma* sp. Anisakid nematodes have often been used as biological tags in fish stock identification studies (MacKenzie et al. 2008, Sequeira et al. 2010, Garcia et al. 2011, among others), and are recognized as being particularly appropriate for this purpose, due to their prolonged permanence in the host. Within the anisakid nematodes, both *Anisakis simplex* s.s. and *Anisakis pegreffii*, belonging to the *Anisakis simplex* s.l. complex, presented significant differences in relative abundance between mainland localities. These differences, however, did not follow any pattern or gradient, like the one observed for example by Mattiucci et al. (2008) in *Trachurus trachurus* from the same region. Furthermore, these species are not reproductively isolated (Martín-Sánchez et al. 2005), producing *A. simplex* s.s. x *A. pegreffii* hybrids, which were also encountered in blackspot seabream both from mainland waters and from the Azores (Hermida et al. 2012), so their taxonomic status is unclear at present. *A. simplex* s.l., in contrast, presented uniformly high relative abundance in all mainland localities and significant differences in both archipelagos, making it a potentially useful biological tag for *P. bogaraveo* in the north-east Atlantic, in addition to *A. physeteris* and *Anisakis* sp. PB-2010.

Juvenile acanthocephalans are also widely recognized as appropriate parasite tags. Although acanthocephalans tend to be short-lived as adults, they have much longer life spans in intermediate and paratenic hosts (Kennedy 1985, 2006). Furthermore, encysted acanthocephalans may remain in an identifiable form in the host even after they die, since the hard structures (spines and hooks) are still recognizable. Moreover, although acanthocephalans are known to affect the behaviour of invertebrate intermediate hosts in order to facilitate transmission, there is no evidence of any effect on the behaviour of vertebrate paratenic hosts; nor do they normally cause any severe pathology in these hosts (Kennedy 2006). Indeed, many stock identification studies have successfully used juvenile acanthocephalans such as *Corynosoma* sp. (Timi & Lanfranchi 2009, McClelland & Melendy 2011) and *Bolbosoma vasculosum* (Santos et al. 2009) as biological tags. Since *Bolbosoma* sp. presented significant differences in prevalence between mainland/Madeira and the Azores, it was also considered a good biological tag for blackpot seabream.

Although the life span of adult digeneans in their fish hosts is not well known, it is generally accepted that most digeneans survive in their definitive hosts for at least several months

(Sandground 1936), which makes them acceptable as biological tags. Also, being endoparasites they are relatively sheltered from sudden changes in abiotic conditions that might be encountered if the fish migrate to different areas. Therefore they have also been used by some authors as biological tags (e.g. Boje et al. 1997). Prevalence of the zoogonid *D. vividum* was higher in mainland waters, though not significantly different from Madeira, but its absence from the Azores make it significantly different from the other two regions. For these reasons, this digenean was considered appropriate as a tag.

Ectoparasites, on the contrary, are more vulnerable to water temperature and other abiotic conditions, and some species can easily be lost from the host. The isopod *Gnathia* sp. is a temporary parasite that is only parasitic during larval stages of its life cycle, and can easily detach from the host (Hadfield et al. 2009); therefore it was discarded as a potential biological tag. The life span of the monogenean *L. virgula* is unknown, but monogeneans generally tend to live less than a year (Rohde 1993), rendering it unsuitable for this purpose. The life span of the copepod *H. pagellibogneravei* is likewise unknown, but most copepods do not live longer than a year (Jones 1998), so it is safer to disregard it as a biological tag for the purpose of stock identification.

Considering all the information on differences in prevalence values and adequacy as biological tags for stock identification, the parasite species selected as biological tags for blackspot seabream in the north-east Atlantic were *Diphterostomum vividum* (Digenea: Zoogonidae), *Anisakis simplex* s.l., *A. physeteris*, *Anisakis* sp. PB-2010 (Nematoda: Anisakidae), and *Bolbosoma* sp. (Acanthocephala: Polymorphidae).

Biogeographical patterns of distribution at the regional level

The mainland sample was clearly separated from both Madeira and the Azores by significantly higher relative abundance of *A. simplex* s.l., and significantly lower prevalence of *Bolbosoma* sp. In addition, mainland samples were characterized by higher prevalences of *D. vividum,* although this was only significantly different from the Azorean sample. The Madeiran sample was characterized by the occurrence, and very high relative abundance, of *Anisakis* sp. PB-2010, an anisakid species that occurred nowhere else, and also by the significantly lower relative abundance of *A. simplex* s.l. when compared to both Azores and the mainland. The Azorean sample was mainly characterized by the significantly higher relative abundance of *A. physeteris.* The prevalence values of *D. vividum* and *A. simplex* s.l. clearly distinguished the Azorean sample from both Madeiran and mainland samples, whereas *Bolbosoma* sp. distinguished it only from the mainland, and the absence of *Anisakis* sp. PB-2010, from the Madeiran sample.

The higher prevalence of *D. vividum* in mainland samples is likely to reflect differences in benthic fauna. The distribution of trematodes is conditioned first and foremost by the distribution of their intermediate hosts, for which they may be strongly specific. It is likely that some benthic invertebrates that act as intermediate hosts of digeneans are more abundant in the continental shelf and slope when compared with oceanic environments. The zoogonid *D. vividum* is strictly specific to *P. bogaraveo* and thus far has only been reported from the British Isles (Nicoll 1912, 1914, Bray 1986, Bray & Gibson 1986). Its intermediate hosts are unknown. However its geographical range does not seem to extend to Azorean waters.

The geographical distribution of anisakid nematodes is conditioned not only by the availability of intermediate hosts, but especially by the presence of appropriate definitive hosts in which the parasite can complete its life cycle. Different species of marine mammals serve as definitive hosts of *Anisakis* spp., and the distribution of the former necessarily restricts the distribution of the latter. The definitive host of *A. physeteris* is the sperm whale, *Physeter macrocephalus*, of which there is a large and stable population in the Azores (Pinela et al. 2009), which would explain the significantly higher relative abundance of this anisakid in that region (Hermida et al. 2012). Significantly higher prevalence of this anisakid has also been detected in *Helicolenus dactylopterus* from the Azores when compared to both Madeira and mainland waters (Sequeira et al. 2010).

The high infection levels by *A. simplex* s.l. seem to be characteristic of the Atlantic part of the Iberian Peninsula, and have been detected in numerous other fish from this region (Abollo et al. 2003, Marques et al. 2006, Mattiucci et al. 2008, Sequeira et al. 2010). The significantly lower relative abundance of this species complex in fish from the Azores, and the again significantly lower relative abundance in Madeira, makes it a good indicator of *P. bogaraveo* provenance.

Particularly striking, in the case of the Madeiran sample, were the very high levels of infection by the anisakid nematode *Anisakis* sp. PB-2010, which was not detected elsewhere, but showed remarkable genetic similarity, and is probably conspecific with, *Anisakis* sp. HC-2005, detected by Kijewska et al. (2009) in fish from the African shelf (Hermida et al. 2012). The definitive host of *Anisakis* sp. PB-2010 is unknown, but a variety of marine mammals are present in the waters around Madeira (Santos-Reis & Mathias 1996), which also explains the higher diversity of anisakid nematodes in this area (Hermida et al. 2012). In any case, its absence from the other localities suggests a restricted distribution of this nematode, which might not be able to complete its life cycle elsewhere, making it an excellent indicator species for *P. bogaraveo* from this region.

Finally, the juvenile acanthocephalan *Bolbosoma* sp. was significantly more prevalent in both archipelagos when compared to mainland waters. Santos et al. (2009) found a significantly higher prevalence of *Bolbosoma vasculosum* in the black scabbardfish, *Aphanopus carbo*, from those two archipelagos when compared with mainland waters, and Shukhgalter (2004) detected

Bolbosoma sp. in *Scomber japonicus* from the Azores, but not in Morocco or Mauritania. This suggests that *Bolbosoma* sp. is mostly present in oceanic habitats, and less frequently on the continental shelf and slope. This might be due to the distribution patterns of its intermediate hosts, which are thought to be pelagic crustaceans (Costa et al. 2000).

Insight into blackspot seabream stocks

Prevalence values of parasite tags within mainland waters were remarkably homogeneous, with only three species exhibiting significant differences between some mainland localities, and these evidenced no clear pattern of distribution. On the contrary, differences between mainland waters, Madeira, and the Azores were clear and significant, with several species from different taxonomic groups exhibiting significant differences in occurrence/prevalence. Taken in conjunction, these results clearly point to the existence of three stocks of *P. bogaraveo* in the studied area: one in mainland Portugal, one in the Azores, and another in Madeira.

The separation of the Azorean population from those from mainland and Madeiran waters is partly backed by genetic studies. Even though there seems to be very little genetic variability in this species, possibly due to a past bottleneck (Bargelloni et al. 2003, Stockley et al. 2005, Patarnello et al. 2007), Stockley et al. (2005) detected significant, albeit low, genetic differentiation in mitochondrial DNA between populations at a regional level, but only between the Azores vs. mainland and Madeira. The dispersal of benthopelagic fish is limited by the existence of topographical barriers, and the ocean basin that lies between the European continental shelf and the Azores archipelago is likely to constitute a formidable obstacle for *P. bogaraveo* (Piñera et al. 2007), whereas Madeira is closer to the mainland. Moreover, the existence of several seamounts that can act as 'stepping stones' for benthopelagic species (Gubbay 2003) is likely to facilitate contact between these two regions. Thus the lack of genetic differentiation between *P. bogaraveo* populations from Madeira and the mainland is not surprising.

Lack of genetic differentiation, however, does not necessarily imply panmixia, especially in a species with unusually low levels of genetic variability. Even a small number of migrant fish per generation can be sufficient to prevent detectable heterogeneity in neutral genetic markers (Carvalho & Hauser 1994). Yet fish populations that are not strictly reproductively isolated can still constitute appropriate fishery management units, responding independently to the effects of exploitation (Pawson & Jennings 1996). The results of this study point to the existence of separate *P. bogaraveo* stocks in Madeira and mainland Portugal. Furthermore, the species with the highest prevalence in Madeira, the copepod *H. pagellibogneravei*, was completely absent from mainland samples. Although copepods may be short-lived and therefore inadequate as biological tags of stock separation, they can nevertheless be useful as indicators of host migrations, or lack thereof (Lester 1990, Moore 2001), and its absence from mainland samples seems to indicate a lack of migration, at least from Madeira to mainland waters, thus corroborating the separation between those two regions inferred from other parasite species.

In conclusion, differences in parasite prevalence in blackspot seabream from the Portuguese EEZ point to the existence of three separate stocks, one in the Azores region (ICES Area X), one in continental shelf/slope waters (ICES Area IXa), and a third in the waters around Madeira (sub-area 1.2 of FAO 34, central-eastern Atlantic).

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Table 1. Prevalence of parasites of *Pagellus bogaraveo* from Portuguese waters and statistically significant differences between localities and/or regions detected by Chi-square test (similar letters indicate no statistical differences).

Parasites	**Matosinhos** (N = 42)	**Figueira da Foz** (N = 92)	**Peniche** (N = 42)	**Sagres** (N = 30)	**Total Mainland**	**Madeira** (N = 56)	**Azores** (N= 86)	**Overall**	**Significant differences**
MYXOSPOREA									
Ceratomyxa sp.	2.4	0.0	4.8	3.3	1.9	1.8	0.0	1.4	
MONOGENEA									
Choricotyle chrysophryi	4.8	1.1	2.4	6.7	2.9	0.0	0.0	1.7	
Lamellodiscus virgula	9.5	2.2	11.9	30.0	9.7	1.8	1.2	6.3	
					b	ab	a		3 reg: p=0.007
DIGENEA									
Accacladocoelium petasiporum	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.3	
Brachyenteron helicoleni	7.1	1.1	23.8	10.0	8.3	0.0	0.0	4.9	
Derogenes varicus	0.0	2.2	0.0	0.0	1.0	0.0	1.2	0.9	
Diphterostomum vividum	14.3	17.4	23.8	23.3	18.9	8.9	0.0	12.6	
	ab	ab	b	ab		a	c		6 loc: p=0.000
					a	a	b		3 reg: p= 0.000
Glomericirrus macrouri	0.0	1.1	0.0	0.0	0.5	0.0	0.0	0.3	
Hemiurus communis	4.8	1.1	0.0	0.0	1.5	0.0	0.0	0.9	
Hemiuridae gen. sp.	0.0	1.1	0.0	3.3	1.0	0.0	0.0	0.6	
Lecithocladium excisum	9.5	29.3	7.1	26.7	20.4	0.0	1.2	12.4	
	ab	c	bd	ac		e	de		6 loc: p=0.000
					b	a	a		3 reg: p=0.000
Lepocreadium album	7.1	10.9	2.4	0.0	6.8	0.0	1.2	4.3	
Opecoelidae gen. sp.	2.4	0.0	0.0	0.0	0.5	0.0	0.0	0.3	
Pachycreadium carnosum	0.0	0.0	0.0	0.0	0.0	1.8	1.2	0.6	
Pycnadenoides senegalensis	2.4	3.3	11.9	0.0	4.4	0.0	1.2	2.9	
CESTODA									
Tetraphyllidea gen. sp.	0.0	0.0	2.4	0.0	0.5	1.8	1.2	0.9	

NEMATODA									
Anisakidae	100.0	90.2	100.0	73.3	91.7	89.3	47.7	80.5	
	a	b	a	c		bc	d		6 loc: p=0.000
					a	a	b		3 reg: p=0.000
Camallanus sp.	0.0	0.0	2.4	0.0	0.5	0.0	0.0	0.3	
Philometra filiformis	0.0	1.1	0.0	0.0	0.5	0.0	0.0	0.3	
ACANTHOCEPHALA									
Bolbosoma sp.	0.0	2.2	4.8	3.3	2.4	8.9	16.3	6.9	
					a	b	b		3 reg: p=0.000
Rhadinorhynchus pristis	2.4	1.1	4.8	3.3	2.4	0.0	7.0	3.2	
CRUSTACEA									
Aega antillensis	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.3	
Aega deshaysiana	0.0	0.0	0.0	0.0	0.0	7.1	0.0	1.1	
Argulus sp.	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.3	
Gnathia sp.	0.0	10.9	0.0	0.0	4.9	21.4	20.9	11.5	
					a	b	b		3 reg: p=0.000
Hatschekia pagellibogneravei	0.0	0.0	0.0	0.0	0.0	96.4	5.8	17.0	
	a	a	a	a		b	a		6 loc: p=0.000
					a	c	b		3 reg: p=0.000
Peniculus fistula	0.0	1.1	0.0	3.3	1.0	0.0	0.0	0.6	
Rocinela danmoniensis	0.0	1.1	2.4	0.0	1.0	5.4	0.0	1.4	

CHAPTER 5

General Discussion, Conclusions and Future Perspectives

GENERAL DISCUSSION

Parasite fauna of *Pagellus bogaraveo*

In the present study, thirty-seven parasite taxa were detected in *Pagellus bogaraveo*, including twenty-four new records for this host: *Accacladocoelium petasiporum* (Digenea: Accacoeliidae), *Brachyenteron helicoleni* (Digenea: Zoogonidae), *Glomericirrus macrouri*, *Lecithocladium excisum* (Digenea: Hemiuridae), *Lepocreadium album* (Digenea: Lepocreadiidae), *Pachycreadium carnosum* (Digenea: Opecoelidae), cestode tetraphyllidean larvae, *Anisakis simplex* x *A. pegreffii* hybrids, *A. ziphidarum*, *A. typica*, *A. physeteris*, *Anisakis* sp. PB-2009, *Anisakis* sp. PB-2010, *Contracaecum* sp. PB-2010, *Hysterothylacium* sp. PB-2010 (Nematoda: Anisakidae), *Camallanus* sp. (Nematoda: Camallanidae), *Philometra filiformis* (Nematoda: Philometridae), *Bolbosoma* sp. (Acanthocephala: Polymorphidae), *Rhadinorhynchus pristis* (Acanthocephala: Rhadinorhynchidae), *Aega antillensis*, *A. deshaysiana*, *Rocinela danmoniensis* (Isopoda: Aegidae), *Gnathia* sp. (Isopoda: Gnathiidae), and *Argulus* sp. (Branchiura: Argulidae). A checklist of the metazoan parasite fauna of *P. bogaraveo* is presented in appendix A.

Generally, the parasite fauna of blackspot seabream was found to be diverse, the most speciose taxonomic groups being the digenea and the nematoda. The diversity of digeneans detected, as well as the near-absence of cestodes, may indicate a preponderance of benthic feeding, since digeneans rely on benthic invertebrates as intermediate hosts, whereas cestodes are usually transmitted pelagically (Marcogliese 2002). This parasite fauna is in sharp contrast with that of a pelagic fish sampled from Portuguese waters (including Madeira, Azores, and one mainland locality), the black scabbardfish, *Aphanopus carbo*, in which a variety of cestodes was detected, and only one digenean species occurred (Santos et al. 2009).

The very high infection levels of juvenile parasites (especially anisakid nematodes), and the relatively lower infection levels of adult parasites, are a common feature of deep-water species (Rohde 1993). Parasite diversity was globally higher in *P. bogaraveo* than in another deep-sea fish, the bluemouth (or blackbelly rosefish), *Helicolenus dactylopterus*, from the two Portuguese Atlantic archipelagos and one locality in mainland waters (Sequeira et al. 2010). Although these two species share many habitat features and are even targeted by the same fisheries, *H. dactylopterus* inhabits greater depths (Menezes et al. 2006), which might explain the lower parasite diversity detected in that fish, since parasite diversity tends to decline with increasing depth (Marcogliese 2002).

However, the hypothesis that these differences are an artifact of sample size cannot be discarded, since both the number of fish observed and the number of sampled localities were higher in the present study.

The proportion of species from each taxonomic group detected in *P. bogaraveo* samples from mainland waters was roughly comparable to those detected in another sparid fish, the bogue, *Boops boops*, in Galicia (Pérez-del Olmo et al. 2007b), and several parasites were shared by both fish, most notably the generalist hemiuroid digeneans *Hemiurus communis*, *Lecithocladium excisum*, and *Derogenes varicus*. Infection levels, however, were very different, with *B. boops* having much higher digenean prevalences, and *P. bogaraveo* having much higher prevalences of anisakid nematodes which, in samples from mainland waters, were mostly constituted by *Anisakis simplex* s.l. While differences in occurrence are likely to be related to the availability of those species in the region, wide differences in infection levels are more likely to reflect the different feeding habits of the two fishes since, unlike *P. bogaraveo*, *B. boops* is a suction-feeder without active prey selectivity (Morato et al. 2001, Pérez-del Olmo et al. 2007a).

Biogeographical differences in parasite assemblages

Within mainland waters, there was remarkable homogeneity in the overall parasite assemblages, whereas wide differences in the infection levels of some parasite groups were detected between mainland waters, Madeira, and the Azores. Prevalence values of the different taxonomic parasite groups are presented in Fig. 15.

High prevalence values of nematodes were detected, almost all of which were anisakids (only a specimen of *Camallanus* sp. and another of *Philometra filiformis* were detected). Nematodes were the most prevalent group in all localities except in Madeira, where crustacean ectoparasites were even more prevalent. This was mainly a reflection of the very high prevalence of the copepod *H. pagellibogneravei* in that region. In any case, the higher ectoparasite infection levels in this subtropical region is in accordance with previous findings of increased abundance of ectoparasites of marine teleosts with decreased latitude (Rohde & Heap, 1998), and is mostly related to differences in water temperature range.

Digeneans were the second most prevalent group in all mainland localities. Digenean prevalence was relatively uniform within mainland waters, and significantly higher in this region than in either archipelago. Among digeneans, the hemiurid *Lecithocladium excisum* exhibited a significantly higher prevalence in mainland waters, much lower in the Azores, and was absent from Madeira. Significantly higher prevalence of *L. excisum* in continental shelf regions has likewise been detected in chub mackerel, in which this parasite was present with relatively high infection levels in mainland waters of Morocco and Mauritania, much lower infection levels in the Great Meteor Bank, and was absent from the Irving Bank, Madeira, and the Canary Islands (Shukhgalter 2004, Oliva et al. 2008, Costa et al. 2011). Sequeira et al. (2010) also detected *Lecithocladium* sp. in *H. dactylopterus* from mainland Portugal, but not from Madeira or the Azores. These differences are likely to be related to greater availability in mainland waters of harpacticoid copepods and polychaetes, which can be used as intermediate hosts for this species (Køie 1991).

Diphterostomum vividum had a significantly higher prevalence in the mainland, lower in Madeira, and was absent from the Azores, and the other zoogonid detected, *B. helicoleni,* occurred in all mainland samples but not in the archipelagos. These two zoogonids had heretofore only been reported from British waters (Nicoll 1912, 1914, Bray 1986, Bray & Gibson 1986, Bray & Kutcha 2006). *Diphterostomum vividum* is a specific parasite of *P. bogaraveo,* whereas *B. helicoleni* had only been detected in another deep-water fish, *H. dactylopterus* (Bray & Kutcha 2006), although, interestingly, it seems to be absent from that host in Portuguese waters (Sequeira et al. 2010). Other digenean species also presented differences in occurrence, but infection levels were too low for these differences to be informative.

Monogenean prevalence was also significantly higher in the mainland than in both Madeira and the Azores. This might be related to higher eutrophication levels of mainland waters, which seem to promote monogenean proliferation, as was also found by Pérez-del Olmo et al. (2007b) in *Boops boops* from Galicia, Spain. However, monogenean prevalence was somewhat variable between mainland localities. Sagres had the highest prevalence of monogeneans, which was mainly due to a higher prevalence of the diplectanid *Lamellodiscus virgula* in this locality. This species is common in the Mediterranean (Oliver 1973, Kaouachi et al. 2010), and its higher prevalence in Sagres might reflect some Mediterranean influence in this southern Portuguese locality. The other monogenean detected, *Choricotyle chrysophryi*, occurred in all mainland localities and in none of the archipelagos, but with uniformly low prevalences.

Prevalence of most parasite groups was invariably lower in the Azores than elsewhere, with the exception of crustaceans, which were more prevalent than in mainland waters, although much less than in Madeira, and acanthocephalans, which had the highest prevalence in the Azores. In the latter case, this might be due to differences in feeding habits of *P. bogaraveo* which, in turn, are likely related to biogeographical differences in food web structure in that oceanic region.

When comparing the proportion of species of each parasite taxonomic group in *P. bogaraveo* from the six localities (Fig. 16), a latitudinal gradient in the proportion of digenean and nematode species was detected. Although the latitudinal range of this study is moderate (less than 10º of maximum difference), the study area is a transitional biogeographical region, from temperate to subtropical.

Portuguese waters are recognized as an important transitional area in the biogeography of fish parasites (Marques et al. 2009). In the present study, the proportion of digenean species was found to decrease with decreasing latitude, and a corresponding increase in the proportion of nematode species was observed. Both these tendencies have been observed by Poulin & Leung (2011) in a meta-analysis of a wide variety of hosts. Whereas the increase in the proportion of nematodes towards lower latitudes can be explained by the increase in diversity of hosts of nematodes in warmer waters (Poulin & Leung 2011), the corresponding decrease of trematode species, despite the increase in mollusc diversity, hence in potential intermediate hosts, is highly unexpected, but Poulin & Leung (2011) suggest that it might be related to latitudinal variation in host specificity. In fact, many digeneans of marine fish have more restricted host ranges in warmer seas (Rohde 1993).

The diversity of anisakid species of *P. bogaraveo* also evidenced biogeographical patterns of distribution. Madeira, in particular, was found to be a hotspot of anisakid nematode diversity, with eight species detected, which is in accordance with other studies from that region (Pontes et al. 2005, Sequeira et al. 2010) and from mainland east African waters at similar latitudes (Farjallah et al. 2008, Kijewska et al. 2009), but greatly increases the number of species known from Madeiran waters, and especially from *P. bogaraveo,* when compared to previous studies such as Costa et al. (2004). Within mainland waters, there was also a modest increase in anisakid nematode diversity from north to south.

Parasite tags and the structure of blackspot seabream populations

Following the criteria outlined in Lester (1990), MacKenzie & Abaunza (1998, 2005), and Lester & MacKenzie (2009), parasite species that: a) evidenced significant differences in infection levels within the study area, b) are known to remain in the host for long periods of time, and c) do not typically cause severe pathology, were selected as adequate parasite tags of *P. bogaraveo* in the north-east Atlantic. These included the digenean *D. vividum,* the anisakid nematodes *A. simplex* s.l., *A. physeteris,* and *Anisakis* sp. PB-2010, and the juvenile acanthocephalan *Bolbosoma* sp.

Prevalence values of these species within mainland waters were remarkably homogeneous, while differences between mainland waters, Madeira, and the Azores, were clear and significant. These results clearly point to the existence of three stocks of *P. bogaraveo* in the studied area: one in mainland Portuguese waters (ICES region IXa), one in the Azores region (ICES Area X), and another in Madeiran waters (sub-area 1.2. of FAO area 34).

The separation of the Azorean *P. bogaraveo* stock from Madeiran and mainland waters is partly backed by genetic studies (Stockley et al. 2005), and is consistent with the limited dispersal of benthopelagic fish. Furthermore, the ocean basin that separates the Azores from European mainland waters is likely to constitute an important obstacle, whereas Madeira is much closer to mainland waters, and the existence of several seamounts between Madeira and the mainland that can act as 'stepping stones' for benthopelagic species (Gubbay 2003) is likely to favour genetic homogeneity between these two regions, considering that even a small number of migrants per generation can be sufficient to prevent detectable heterogeneity in neutral genetic markers (Carvalho & Hauser 1994). Nevertheless, fish populations that are not strictly reproductively isolated can still constitute appropriate fishery management units that respond independently to the effects of exploitation (Pawson & Jennings 1996), and results from this study, especially concerning the anisakids *Anisakis simplex* s.l. and *Anisakis* sp. PB-2010, point to a clear separation between Madeiran and mainland *P. bogaraveo* populations. Furthermore, although *H. pagellibogneravei* does not fulfill all the necessary requirements to be used as a tag for stock identification purposes since its life-span on the host is unknown, it can nevertheless be used as an indicator of fish migrations and, in the present study, its complete absence from mainland waters strongly suggests a lack of migration of blackspot seabream, at least from Madeira to the mainland.

These conclusions are also corroborated by the detection of a latitudinal gradient in the proportion of digenean and nematode species in *P. bogaraveo* from the sampled localities. Latitudinal patterns in parasite distribution can be useful for the study of fish populations (Timi 2003) and, in the present study, the gradients detected in the proportion of species from different parasite groups seem to suggest limited latitudinal movement on the part of blackspot seabream.

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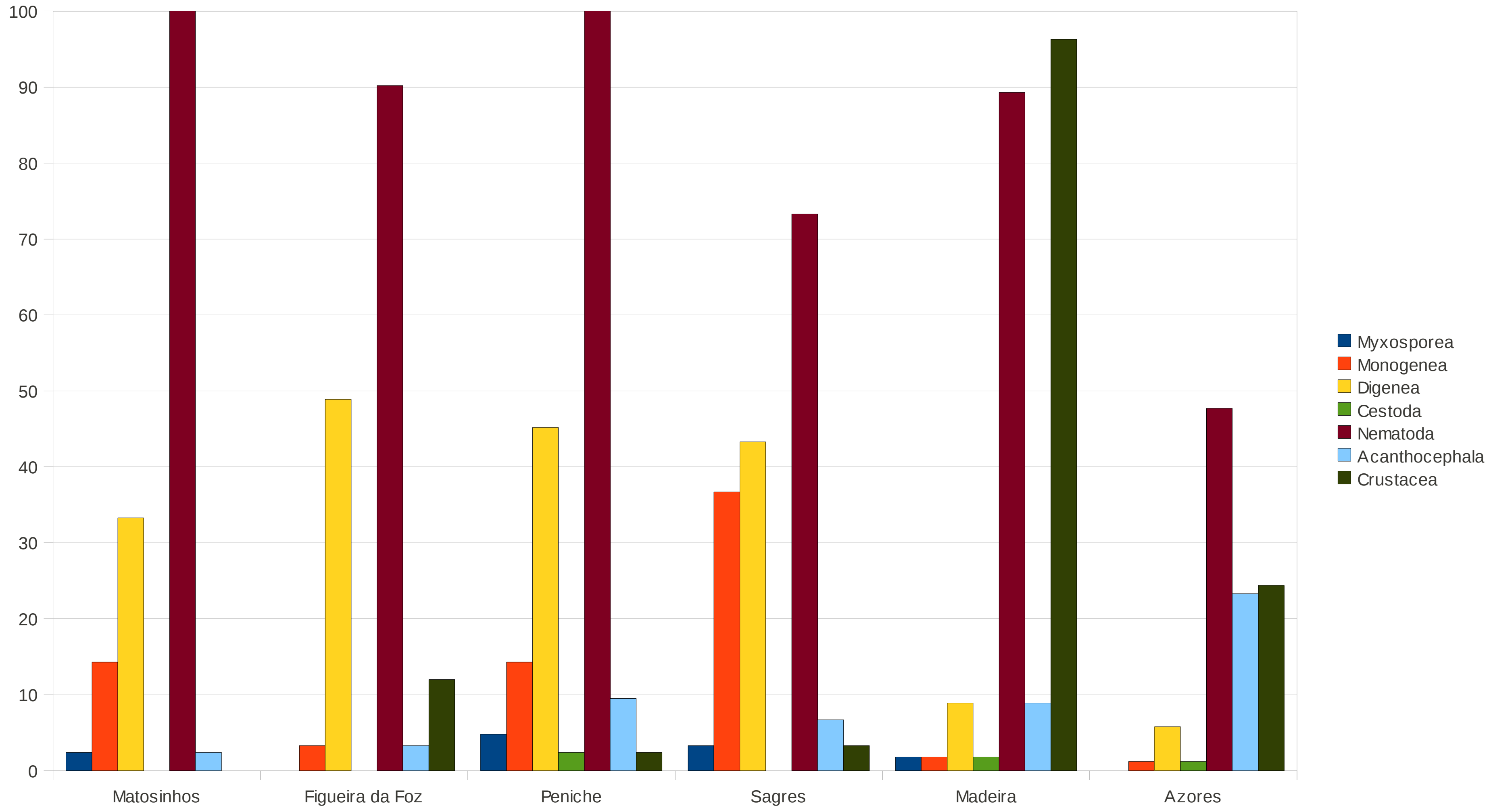

Fig. 15. Prevalence (%) of major taxonomic parasite groups in *P. bogaraveo* from the studied localities.

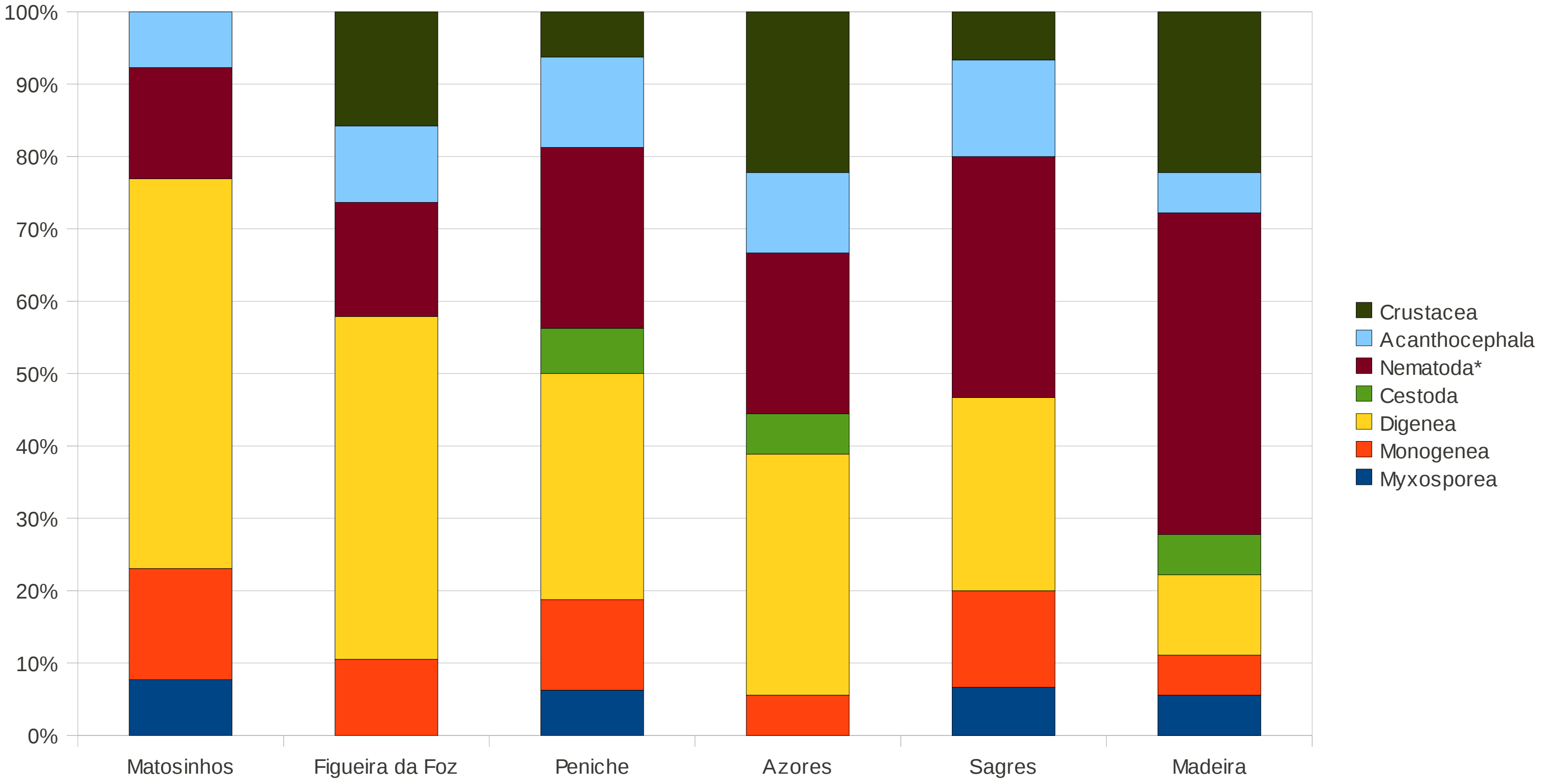

Fig. 16. Proportion of parasite species (%) of each major taxonomic group in parasite assemblages of *Pagellus bogaraveo* from different localities in Portuguese waters. Samples are ordered according to approximate latitude, from north to south (Matosinhos 41º18'N, Figueira da Foz 40º15'N, Peniche 39º36'N, Ponta Delgada 37º73' N, Sagres 37ºN, Funchal 32º63' N). **A. simplex* and *A. pegreffii* were considered as two different species; *A. simplex* x *A. pegreffii* hybrids were excluded from this analysis.

CONCLUSIONS

- This was the first comprehensive study on the parasite fauna of blackspot seabream, *Pagellus bogaraveo* (Brünnich, 1768), an important sparid fish that is targeted mostly by Portuguese and Spanish fisheries in the north-east Atlantic.

- Thirty-seven parasite taxa were detected, including twenty-four new records in this host: *Accacladocoelium petasiporum* (Digenea: Accacoeliidae), *Brachyenteron helicoleni* (Digenea: Zoogonidae), *Glomericirrus macrouri*, *Lecithocladium excisum* (Digenea: Hemiuridae), *Lepocreadium album* (Digenea: Lepocreadiidae), *Pachycreadium carnosum* (Digenea: Opecoelidae), cestode tetraphyllidean larvae, *Anisakis simplex* x *A. pegreffii* hybrids, *A. ziphidarum*, *A. typica*, *A. physeteris*, *Anisakis* sp. PB-2009, *Anisakis* sp. PB-2010, *Contracaecum* sp. PB-2010, *Hysterothylacium* sp. PB-2010 (Nematoda: Anisakidae), *Camallanus* sp. (Nematoda: Camallanidae), *Philometra filiformis* (Nematoda: Philometridae), *Bolbosoma* sp. (Acanthocephala: Polymorphidae), *Rhadinorhynchus pristis* (Acanthocephala: Rhadinorhynchidae), *Aega antillensis*, *A. deshaysiana*, *Rocinela danmoniensis* (Isopoda: Aegidae), *Gnathia* sp. (Isopoda: Gnathiidae), and *Argulus* sp. (Branchiura: Argulidae).

- A study on the ectoparasite communities of blackspot seabream revealed the existence of several species that might have a negative impact in aquaculture; however ectoparasite infection levels were much higher in Madeira, suggesting that aquaculture of *P. bogaraveo* might experience less difficulty in colder, mainland waters.

- Microhabitat preferences were observed in the distribution of the copepod *Hatschekia pagellibogneravei* in the gills of *P. bogaraveo*, and also in several gastrointestinal helminth species.

- Molecular identification of anisakid nematodes of *P. bogaraveo* from Portuguese waters resulted in the observation of ten different PCR-RFLP patterns of the ITS region, four of which were novel, and phylogenetic analysis permitted the placement of new anisakid species detected in Madeira within the known phylogenetic structure of *Anisakis* spp.

- An in-depth ecological study of the gastrointestinal helminth communities of blackspot seabream indicated that these communities were more diverse, even, and stable in mainland waters than in both archipelagos, with *P. bogaraveo* from Madeira exhibiting very impoverished gastrointestinal helminth communities, and the Azorean region exhibiting a remarkable diversity of helminth species, all of which with extremely low infection levels.

- Regional differences in parasite assemblages were detected between mainland waters, Madeira, and the Azores. Digeneans and monogeneans were more prevalent in mainland waters, whereas crustaceans were more prevalent in Madeira, and *P. bogaraveo* from the Azores exhibited the highest prevalence of acanthocephalans, but generally lower infection levels. Myxosporeans and cestodes exhibited very low infection levels overall.

- The parasite assemblages of *P. bogaraveo* were diverse, with the most speciose groups being the Nematoda and Digenea. The parasite fauna of blackspot seabream mainly reflects the influence of its habitat and feeding habits.

- Latitudinal gradients were detected in the proportion of digenean and nematode species. The former increased with latitude, while the latter decreased. The region of Madeira, in particular, was found to be a hotspot of anisakid nematode diversity, with eight anisakid species detected in that region.

- The species *Diphterostomum vividum* (Digenea: Zoogonidae), *Anisakis simplex* s.l., *A. physeteris, Anisakis* sp. PB-2010 (Nematoda: Anisakidae), and *Bolbosoma* sp. (Acanthocephala: Polymorphidae) were selected as adequate biological tags for the study of *P. bogaraveo* stocks in the north-east Atlantic according to the following criteria: existence of significant differences in prevalence within the study area, long permanence on the host, and lack of serious pathogenic effects on the host.

- Differences in prevalence of these species indicated the existence of three separate *P. bogaraveo* stocks in the north-east Atlantic: one in the Azores region (ICES Area X), one in continental Portuguese shelf/slope waters (ICES Area IXa), and a third in the waters around Madeira (sub-area 1.2 of FAO area 34).

FUTURE PERSPECTIVES

Several interesting questions have arisen from this study, which might be profitably elucidated in future research.

- The existence of three *P. bogaraveo* stocks in the north-east Atlantic needs to be tested through the use of additional, complementary techniques. Otoliths and muscle samples were collected from the specimens observed in this study and efforts are being undertaken to ensure that this material will be utilized for otolith microchemistry and genetic analysis in the near future.

- The hypothesis that the low infection levels by gastrointestinal helminths and, in particular, digeneans, in *P. bogaraveo* from Madeira are due to a shift to a more pelagic diet in subtropical waters needs to be further investigated by conducting comparative studies of its feeding habits in different regions since, at present, the only study available on the feeding habits of blackspot seabream is from the Azores.

- Sampling of *P. bogaraveo* individuals in future parasitological studies should be extended to include small juvenile fish and very large, older individuals. Size range was somewhat restricted in the present study for practical and economic reasons, but parasite studies of a more diverse array of ontogenetic stages might provide a more complete picture of the ecological and biological factors behind the acquisition of different parasites.

- There is a dearth of parasitological studies of sparid fish not only in the north-east Atlantic, but especially in Portuguese waters. Some species detected in this study (e.g. *Hatschekia pagellibogneravei*) are known to infect other sparids, yet at present there is no information regarding their occurrence in other sparid fishes in Portuguese waters. This is important not only from an ecological perspective but also since this and other ectoparasite species detected here may have a negative impact in sparid aquaculture.

- Sampling of *P. bogaraveo* from the west African coast and the Canary Islands might yield further insight into the stock structure of this species in the central-east Atlantic. The presence in *P. bogaraveo* from Madeira of the anisakid species *Anisakis* sp. PB-2010, which appears to be conspecific with *Anisakis* sp. HC-2005, detected in fish from the west African coast, suggests a similarity in the available parasite species in these two areas, but very few studies on the parasite fauna of *P. bogaraveo* from west African mainland waters have been conducted and none has included molecular identification of anisakid nematodes.

- It is of the utmost importance to conduct studies on the life cycles, intermediate hosts, and longevity in the host of more parasite species, since this information would facilitate the use of more parasite species for the study of fish stocks.

APPENDIX A

Checklist of the metazoan parasites detected in *Pagellus bogaraveo*

Checklist of metazoan parasites of *Pagellus bogaraveo*
(syn. *Pagellus centrodontus*, *Sparus centrodontus*)

Parasite	Area	References
Myxosporea		
Ceratomyxa arcuata	Atlantic	Thélohan 1894
	Mediterranean	Parisi 1912
Ceratomyxa sparusaurati	Mediterranean	Mladineo 2003 Mladineo 2006
Ceratomyxa sp.	Atlantic	Present work
Monogenea		
Family Capsalidae Baird, 1853		
Encotyllabe pagelli van Beneden & Hesse, 1863	Atlantic	van Beneden & Hesse 1864
Family Diclidophoridae Fuhrmann, 1928		
Choricotyle chrysophryi (van Beneden & Hesse, 1863) (syn. *Cyclocotyla chrysophryi*)	Atlantic	Rees & Llewellyn 1941 Llewellyn 1941 Llewellyn 1956 Kaouachi et al. 2010 Hermida et al. *in press*-a
	Mediterranean	Kaouachi et al. 2010
Choricotyle pagelli Llewellyn, 1941	Atlantic	Price 1943
Family Diplectanidae Bychovsky, 1957		
Lamellodiscus virgula Euzet & Oliver, 1967 (syn. *Lamellodiscus obeliae* Oliver, 1973)	Atlantic	Oliver 1973 Kaouachi et al. 2010 Hermida et al. *in press*-a
	Mediterranean	Paggi et al. 1998 Desdevises et al. 2000 Kaouachi et al. 2010
Family Microcotylidae Taschenberg, 1879		
Microcotyle centrodonti Brown, 1929	Atlantic	Brown 1929
Digenea		
Family Accacoeliidae Odhner, 1911		
Accacladocoelium petasiporum Odhner, 1928	Atlantic	Hermida et al. *in press*-b
Family Derogenidae Nicoll, 1910		
Derogenes varicus (Müller, 1784)	Atlantic	Nicoll 1914 Little 1929 Hermida et al. *in press*-b
Family Fellodistomidae		
Steringotrema divergens (Rudolphi, 1809)	Atlantic	Fischthal & Thomas 1972
Steringotrema pagelli (van Beneden, 1871) (syn. *Distoma pagelli* van Beneden, 1870	Atlantic, Mediterranean	van Beneden 1871 Odhner 1911 Nicoll 1914 Little 1929
Family Hemiuridae Looss, 1899		

Parasite	**Area**	**References**
Glomericirrus macrouri (Gaevskaja, 1973)	Atlantic	Hermida et al. *in press*-b
Hemiurus communis Odhner, 1905	Atlantic	Nicoll 1914 Little 1929 Gibson & Bray 1986 Lozano et al. 2001 Hermida et al. *in press*-b
Lecithocladium excisum (Rudolphi, 1819)	Atlantic	Hermida et al. *in press*-b
Parahemiurus merus (Linton, 1910)	Atlantic	Fischthal & Thomas 1972
Hemiuridae gen. sp.	Atlantic	Hermida et al. *in press*-b
Family Lepocreadiidae Odhner, 1905		
Lepocreadium album (Stossich, 1890)	Atlantic	Hermida et al. *in press*-b
Family Opecoelidae Ozaki, 1925		
Pachycreadium carnosum (Rudolphi, 1819)	Atlantic	Hermida et al. *in press*-b
Pycnadenoides senegalensis	Atlantic	Fischthal & Thomas 1972 Gibson & Bray 1982 Bartoli et al. 1989 Hermida et al. *in press*-b
Opecoelidae gen. sp.	Atlantic	Hermida et al. *in press*-b
Family Zoogonidae Odhner, 1902		
Brachyenteron helicoleni Bray & Kutcha, 2006	Atlantic	Hermida et al. *in press*-b
Diphterostomum vividum (Nicoll, 1912) (syn. *Zoogonoides vividus*, *Zoonogenus vividus*)	Atlantic	Nicoll 1912 Nicoll 1914 Bray 1986 Bray & Gibson 1986 Hermida et al. *in press*-b
Cestoda		
Family Lacistorhynchidae Guiart, 1937		
Grillotia smarisgora (Wagener, 1854)	Atlantic	MacKenzie 1990 Palm 2004
Tetraphyllidea incertae sedis		
Tetraphyllidea gen. sp.	Atlantic	Hermida et al. *in press*-b
Nematoda		
Family Anisakidae Skrjabin & Karokhin, 1945		
Anisakis pegreffii Campana-Rouget & Biocca, 1955	Atlantic	Costa et al. 2004 Hermida et al. 2012b
Anisakis physeteris (Baylis, 1923)	Atlantic	Hermida et al. 2012b
Anisakis simplex (Rudolphi, 1809) sensu stricto	Atlantic	Costa et al. 2004 Hermida et al. 2012b
Anisakis simplex x *Anisakis pegreffii*	Atlantic	Hermida et al. 2012b
Anisakis typica (Diesing, 1860)	Atlantic	Hermida et al. 2012b
Anisakis ziphidarum Paggi, Nascetti, Webb, Mattiucci, Cianchi & Bullini, 1988	Atlantic	Hermida et al. 2012b
Anisakis sp. PB-2009	Atlantic	Hermida et al. 2012b
Anisakis sp. PB-2010	Atlantic	Hermida et al. 2012b

Parasite	Area	References
Anisakis sp. (type II larvae)	Atlantic	Costa et al. 2004
Contracaecum sp. PB-2010	Atlantic	Hermida et al. 2012b
Hysterothylacium aduncum (Rudolphi, 1802)	Mediterranean	Mladineo 2006
Hysterothylacium bidentatum (Linstow, 1899) (syn. *Contracaecum bidentatum*)	Atlantic	Oliveira Rodrigues et al. 1975
Hysterothylacium sp. PB-2010	Atlantic	Hermida et al. 2012b
Hysterothylacium sp.	Atlantic	Costa et al. 2004
Family Camallanidae Railliet & Henry, 1915		
Camallanus sp. Railliet & Henry, 1915	Atlantic	Hermida et al. *in press*-b
Family Cucullanidae Cobbold, 1864		
Cucullanus chrysophrydis Gendre, 1928	Atlantic	Campana-Rouget 1957
Family Philometridae Baylis & Daubney, 1926		
Philometra filiformis (Stossich, 1896)	Atlantic	Present work
Acanthocephala		
Family Polymorphidae Meyer, 1931		
Bolbosoma sp. Porta, 1908	Atlantic	Present work
Family Rhadinorhynchidae Travassos, 1923		
*Rhadinorhynchus pristis (*Rudolphi, 1802)	Atlantic	Hermida et al. *in press*-b
Crustacea: Branchiura		
Family Argulidae Leach, 1819		
Argulus sp. Müller, 1785	Atlantic	Hermida et al. *in press*-a
Crustacea: Copepoda		
Family Caligidae Burmeister, 1835		
Caligus centrodonti Baird, 1850 (syn. *C. abbreviatus* Kröyer, 1863)	Atlantic	Baird 1850 Bassett-Smith 1899 Wilson 1905 Norman & Scott 1906 Scott & Scott 1913 Kabata 1979 Kabata 1992
Caligus curtus Müller, 1785	Atlantic	Kabata 1992
Caligus diaphanus von Nordmann, 1832	Atlantic	Thompson 1847 Kabata 1992
	Mediterranean	Raibaut et al. 1998
Caligus ligusticus Brian, 1906	Mediterranean	Raibaut et al. 1998
Caligus minimus Otto, 1821	Mediterranean	Brian 1934 Raibaut et al. 1998
Family Hatschekiidae Kabata, 1979		
Hatschekia pagellibogneravei (Hesse, 1878) (alt. *H. pagellibogueravei*; syn. *H. cornigera*; syn. *Cycnus pagelli bogueravei*)	Atlantic	Hesse 1878 Hesse 1879 Scott 1909 Kabata 1979 Jones 1985 Hermida et al. 2012a

Parasite	Area	References
		Hermida et al. *in press*-a
	Mediterranean	Goggio 1905 Brian 1914 Raibaut et al. 1998 Boualleg et al. 2010
Family Lernaeopodidae Milne-Edwards, 1840		
Alella pagelli (Krøyer, 1863)	Mediterranean	van Beneden 1870 Scott & Scott 1913 Raibaut et al. 1998
Family Pennellidae Burmeister, 1816		
Peniculus fistula von Nordmann, 1832	Atlantic	Gooding 1957 Hermida et al. *in press*-a
Family Philichthyidae Vogt, 1877		
Colobomatus oblatae Delamare Deboutteville & Nunes-Ruivo, 1952 (Richiardi, 1900)	Mediterranean	Raibaut et al. 1998
Colobomatus pagelli (Richiardi, 1877)	Mediterranean	Raibaut et al. 1998
Crustacea: Isopoda		
Family Aegidae White, 1850		
*Aega antillensis S*chiödte & Meinert, 1879	Atlantic	Hermida et al. *in press*-a
Aega deshaysiana (Milne-Edwards, 1840)	Atlantic	Hermida et al. *in press*-a
Rocinela danmoniensis Leach, 1818	Atlantic	Hermida et al. *in press*-a
Family Cymothoidae Leach, 1818		
*Ceratothoa collaris S*chiödte & Meinert, 1883	Mediterranean	Dollfus & Trilles 1976
Family Gnathiidae Leach, 1814		
Gnathia sp. Leach, 1814	Atlantic	Hermida et al. *in press*-a

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APPENDIX B

Supplementary material to Hermida et al. 2012

Parasitol. Res. 110: 1919-1928

Parasitology Research

"Infection levels and diversity of anisakid nematodes in blackspot seabream, *Pagellus bogaraveo*, from Portuguese waters"

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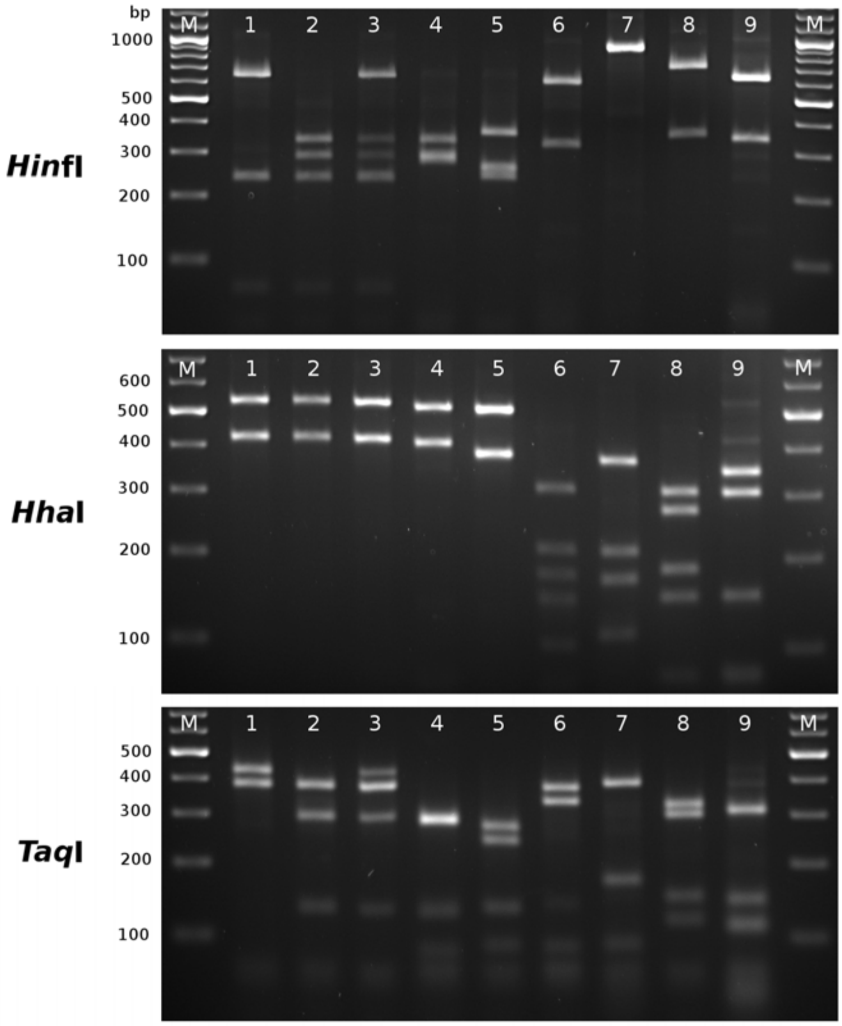

Fig. S1 PCR-RFLP patterns obtained for different anisakid species cutting a ~1Kb fragment (ITS region) with the restriction endonucleases *Hin*fI, *Hha*I, and *Taq*I, 1, *Anisakis simplex* s.s.; 2, *A. pegreffii*; 3, *A. simplex* s.s. x *A. pegreffii*; 4, *A. ziphidarum*; 5, *A. physeteris*; 6, *A. typica*; 7, *Anisakis* sp. PB-2010; 8. *Hysterothylacium* sp.; 9, *Contracaecum* sp.; M, GeneRuler™DNA ladder mix (Fermentas).

Parasitology Research

"Infection levels and diversity of anisakid nematodes in blackspot seabream, *Pagellus bogaraveo*, from Portuguese waters"

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Table S1 Number of base substitutions per site from between sequences and their standard error estimate(s), computed in MEGA5 (Tamura et al., 2011). Analyses were conducted using the Kimura 2-parameter model (Kimura 1980). The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.3185).

Species 1	Species 2	Dist	Std. Err
A physeteris(JN005754)	A physeteris(AY826721)	0,003	0,002
A physeteris(JN005754)	A physeteris(JN005763)	0,005	0,002
A physeteris(AY826721)	A physeteris(JN005763)	0,008	0,003
A physeteris(JN005754)	A brevispiculata(EU624344)	0,054	0,010
A physeteris(AY826721)	A brevispiculata(EU624344)	0,058	0,011
A physeteris(JN005763)	A brevispiculata(EU624344)	0,061	0,011
A physeteris(JN005754)	A paggiae(EU624345)	0,078	0,013
A physeteris(AY826721)	A paggiae(EU624345)	0,082	0,014
A physeteris(JN005763)	A paggiae(EU624345)	0,086	0,014
A brevispiculata(EU624344)	A paggiae(EU624345)	0,063	0,011
A physeteris(JN005754)	A pegreffii(JN005756)	0,348	0,045
A physeteris(AY826721)	A pegreffii(JN005756)	0,357	0,047
A physeteris(JN005763)	A pegreffii(JN005756)	0,365	0,048
A brevispiculata(EU624344)	A pegreffii(JN005756)	0,366	0,051
A paggiae(EU624345)	A pegreffii(JN005756)	0,339	0,045
A physeteris(JN005754)	A pegreffii(EU624343)	0,347	0,045
A physeteris(AY826721)	A pegreffii(EU624343)	0,356	0,047
A physeteris(JN005763)	A pegreffii(EU624343)	0,364	0,048
A brevispiculata(EU624344)	A pegreffii(EU624343)	0,365	0,051
A paggiae(EU624345)	A pegreffii(EU624343)	0,338	0,045
A pegreffii(JN005756)	A pegreffii(EU624343)	0,001	0,001
A physeteris(JN005754)	A pegreffii(JN005765)	0,360	0,047
A physeteris(AY826721)	A pegreffii(JN005765)	0,370	0,049
A physeteris(JN005763)	A pegreffii(JN005765)	0,378	0,050
A brevispiculata(EU624344)	A pegreffii(JN005765)	0,374	0,052
A paggiae(EU624345)	A pegreffii(JN005765)	0,351	0,047
A pegreffii(JN005756)	A pegreffii(JN005765)	0,006	0,003
A pegreffii(EU624343)	A pegreffii(JN005765)	0,005	0,002
A physeteris(JN005754)	A simplex(JN005764)	0,351	0,046
A physeteris(AY826721)	A simplex(JN005764)	0,361	0,047
A physeteris(JN005763)	A simplex(JN005764)	0,369	0,048

A brevispiculata(EU624344)	A simplex(JN005764)	0,360	0,050
A paggiae(EU624345)	A simplex(JN005764)	0,343	0,046
A pegreffii(JN005756)	A simplex(JN005764)	0,004	0,002
A pegreffii(EU624343)	A simplex(JN005764)	0,002	0,002
A pegreffii(JN005765)	A simplex(JN005764)	0,007	0,003
A physeteris(JN005754)	A simplex(EU624342)	0,351	0,046
A physeteris(AY826721)	A simplex(EU624342)	0,361	0,047
A physeteris(JN005763)	A simplex(EU624342)	0,369	0,048
A brevispiculata(EU624344)	A simplex(EU624342)	0,360	0,050
A paggiae(EU624345)	A simplex(EU624342)	0,343	0,046
A pegreffii(JN005756)	A simplex(EU624342)	0,004	0,002
A pegreffii(EU624343)	A simplex(EU624342)	0,002	0,002
A pegreffii(JN005765)	A simplex(EU624342)	0,007	0,003
A simplex(JN005764)	A simplex(EU624342)	0,000	0,000
A physeteris(JN005754)	A simplex(JN005757)	0,359	0,047
A physeteris(AY826721)	A simplex(JN005757)	0,369	0,049
A physeteris(JN005763)	A simplex(JN005757)	0,377	0,050
A brevispiculata(EU624344)	A simplex(JN005757)	0,368	0,051
A paggiae(EU624345)	A simplex(JN005757)	0,350	0,047
A pegreffii(JN005756)	A simplex(JN005757)	0,006	0,003
A pegreffii(EU624343)	A simplex(JN005757)	0,005	0,002
A pegreffii(JN005765)	A simplex(JN005757)	0,010	0,003
A simplex(JN005764)	A simplex(JN005757)	0,002	0,002
A simplex(EU624342)	A simplex(JN005757)	0,002	0,002
A physeteris(JN005754)	A simplexXA pegreffii(JN005768)	0,351	0,046
A physeteris(AY826721)	A simplexXA pegreffii(JN005768)	0,361	0,047
A physeteris(JN005763)	A simplexXA pegreffii(JN005768)	0,369	0,048
A brevispiculata(EU624344)	A simplexXA pegreffii(JN005768)	0,360	0,050
A paggiae(EU624345)	A simplexXA pegreffii(JN005768)	0,343	0,046
A pegreffii(JN005756)	A simplexXA pegreffii(JN005768)	0,005	0,002
A pegreffii(EU624343)	A simplexXA pegreffii(JN005768)	0,004	0,002
A pegreffii(JN005765)	A simplexXA pegreffii(JN005768)	0,008	0,003
A simplex(JN005764)	A simplexXA pegreffii(JN005768)	0,004	0,002
A simplex(EU624342)	A simplexXA pegreffii(JN005768)	0,004	0,002
A simplex(JN005757)	A simplexXA pegreffii(JN005768)	0,006	0,003
A physeteris(JN005754)	A simplex C(AY826722)	0,346	0,045
A physeteris(AY826721)	A simplex C(AY826722)	0,355	0,046
A physeteris(JN005763)	A simplex C(AY826722)	0,363	0,047
A brevispiculata(EU624344)	A simplex C(AY826722)	0,354	0,049
A paggiae(EU624345)	A simplex C(AY826722)	0,337	0,045
A pegreffii(JN005756)	A simplex C(AY826722)	0,006	0,003
A pegreffii(EU624343)	A simplex C(AY826722)	0,005	0,002
A pegreffii(JN005765)	A simplex C(AY826722)	0,010	0,003
A simplex(JN005764)	A simplex C(AY826722)	0,005	0,002
A simplex(EU624342)	A simplex C(AY826722)	0,005	0,002
A simplex(JN005757)	A simplex C(AY826722)	0,007	0,003
A simplexXA pegreffii(JN005768)	A simplex C(AY826722)	0,006	0,003
A physeteris(JN005754)	A ziphidarum(JN005767)	0,336	0,045
A physeteris(AY826721)	A ziphidarum(JN005767)	0,346	0,047
A physeteris(JN005763)	A ziphidarum(JN005767)	0,354	0,048
A brevispiculata(EU624344)	A ziphidarum(JN005767)	0,350	0,048
A paggiae(EU624345)	A ziphidarum(JN005767)	0,312	0,042
A pegreffii(JN005756)	A ziphidarum(JN005767)	0,067	0,011
A pegreffii(EU624343)	A ziphidarum(JN005767)	0,065	0,011

A pegreffii(JN005765)	A ziphidarum(JN005767)	0,070	0,011
A simplex(JN005764)	A ziphidarum(JN005767)	0,067	0,011
A simplex(EU624342)	A ziphidarum(JN005767)	0,067	0,011
A simplex(JN005757)	A ziphidarum(JN005767)	0,070	0,012
A simplexXA pegreffii(JN005768)	A ziphidarum(JN005767)	0,069	0,011
A simplex C(AY826722)	A ziphidarum(JN005767)	0,067	0,011
A physeteris(JN005754)	A ziphidarum(AY826725)	0,331	0,045
A physeteris(AY826721)	A ziphidarum(AY826725)	0,341	0,046
A physeteris(JN005763)	A ziphidarum(AY826725)	0,349	0,047
A brevispiculata(EU624344)	A ziphidarum(AY826725)	0,345	0,048
A paggiae(EU624345)	A ziphidarum(AY826725)	0,308	0,042
A pegreffii(JN005756)	A ziphidarum(AY826725)	0,065	0,011
A pegreffii(EU624343)	A ziphidarum(AY826725)	0,064	0,011
A pegreffii(JN005765)	A ziphidarum(AY826725)	0,069	0,011
A simplex(JN005764)	A ziphidarum(AY826725)	0,065	0,011
A simplex(EU624342)	A ziphidarum(AY826725)	0,065	0,011
A simplex(JN005757)	A ziphidarum(AY826725)	0,069	0,011
A simplexXA pegreffii(JN005768)	A ziphidarum(AY826725)	0,067	0,011
A simplex C(AY826722)	A ziphidarum(AY826725)	0,065	0,011
A ziphidarum(JN005767)	A ziphidarum(AY826725)	0,001	0,001
A physeteris(JN005754)	A ziphidarum(JN005766)	0,331	0,045
A physeteris(AY826721)	A ziphidarum(JN005766)	0,341	0,047
A physeteris(JN005763)	A ziphidarum(JN005766)	0,349	0,047
A brevispiculata(EU624344)	A ziphidarum(JN005766)	0,345	0,048
A paggiae(EU624345)	A ziphidarum(JN005766)	0,308	0,042
A pegreffii(JN005756)	A ziphidarum(JN005766)	0,070	0,011
A pegreffii(EU624343)	A ziphidarum(JN005766)	0,069	0,011
A pegreffii(JN005765)	A ziphidarum(JN005766)	0,074	0,012
A simplex(JN005764)	A ziphidarum(JN005766)	0,071	0,011
A simplex(EU624342)	A ziphidarum(JN005766)	0,071	0,011
A simplex(JN005757)	A ziphidarum(JN005766)	0,074	0,012
A simplexXA pegreffii(JN005768)	A ziphidarum(JN005766)	0,072	0,012
A simplex C(AY826722)	A ziphidarum(JN005766)	0,071	0,012
A ziphidarum(JN005767)	A ziphidarum(JN005766)	0,005	0,002
A ziphidarum(AY826725)	A ziphidarum(JN005766)	0,004	0,002
A physeteris(JN005754)	A nascettii(AY260555)	0,377	0,049
A physeteris(AY826721)	A nascettii(AY260555)	0,388	0,051
A physeteris(JN005763)	A nascettii(AY260555)	0,397	0,051
A brevispiculata(EU624344)	A nascettii(AY260555)	0,381	0,052
A paggiae(EU624345)	A nascettii(AY260555)	0,328	0,042
A pegreffii(JN005756)	A nascettii(AY260555)	0,105	0,016
A pegreffii(EU624343)	A nascettii(AY260555)	0,103	0,016
A pegreffii(JN005765)	A nascettii(AY260555)	0,108	0,016
A simplex(JN005764)	A nascettii(AY260555)	0,106	0,016
A simplex(EU624342)	A nascettii(AY260555)	0,106	0,016
A simplex(JN005757)	A nascettii(AY260555)	0,110	0,016
A simplexXA pegreffii(JN005768)	A nascettii(AY260555)	0,108	0,016
A simplex C(AY826722)	A nascettii(AY260555)	0,108	0,016
A ziphidarum(JN005767)	A nascettii(AY260555)	0,078	0,013
A ziphidarum(AY826725)	A nascettii(AY260555)	0,076	0,013
A ziphidarum(JN005766)	A nascettii(AY260555)	0,082	0,013
A physeteris(JN005754)	Anisakis MP-2005(EU718477)	0,354	0,045
A physeteris(AY826721)	Anisakis MP-2005(EU718477)	0,364	0,047
A physeteris(JN005763)	Anisakis MP-2005(EU718477)	0,372	0,047

A brevispiculata(EU624344)	Anisakis MP-2005(EU718477)	0,385	0,052
A paggiae(EU624345)	Anisakis MP-2005(EU718477)	0,321	0,041
A pegreffii(JN005756)	Anisakis MP-2005(EU718477)	0,102	0,015
A pegreffii(EU624343)	Anisakis MP-2005(EU718477)	0,100	0,015
A pegreffii(JN005765)	Anisakis MP-2005(EU718477)	0,106	0,016
A simplex(JN005764)	Anisakis MP-2005(EU718477)	0,102	0,015
A simplex(EU624342)	Anisakis MP-2005(EU718477)	0,102	0,015
A simplex(JN005757)	Anisakis MP-2005(EU718477)	0,106	0,016
A simplexXA pegreffii(JN005768)	Anisakis MP-2005(EU718477)	0,104	0,015
A simplex C(AY826722)	Anisakis MP-2005(EU718477)	0,104	0,015
A ziphidarum(JN005767)	Anisakis MP-2005(EU718477)	0,079	0,013
A ziphidarum(AY826725)	Anisakis MP-2005(EU718477)	0,077	0,013
A ziphidarum(JN005766)	Anisakis MP-2005(EU718477)	0,083	0,013
A nascettii(AY260555)	Anisakis MP-2005(EU718477)	0,004	0,002
A physeteris(JN005754)	Anisakis PB-2009(JN005758)	0,233	0,029
A physeteris(AY826721)	Anisakis PB-2009(JN005758)	0,240	0,030
A physeteris(JN005763)	Anisakis PB-2009(JN005758)	0,244	0,031
A brevispiculata(EU624344)	Anisakis PB-2009(JN005758)	0,249	0,034
A paggiae(EU624345)	Anisakis PB-2009(JN005758)	0,228	0,031
A pegreffii(JN005756)	Anisakis PB-2009(JN005758)	0,157	0,023
A pegreffii(EU624343)	Anisakis PB-2009(JN005758)	0,157	0,023
A pegreffii(JN005765)	Anisakis PB-2009(JN005758)	0,165	0,023
A simplex(JN005764)	Anisakis PB-2009(JN005758)	0,162	0,023
A simplex(EU624342)	Anisakis PB-2009(JN005758)	0,162	0,023
A simplex(JN005757)	Anisakis PB-2009(JN005758)	0,164	0,023
A simplexXA pegreffii(JN005768)	Anisakis PB-2009(JN005758)	0,159	0,023
A simplex C(AY826722)	Anisakis PB-2009(JN005758)	0,160	0,023
A ziphidarum(JN005767)	Anisakis PB-2009(JN005758)	0,195	0,028
A ziphidarum(AY826725)	Anisakis PB-2009(JN005758)	0,192	0,028
A ziphidarum(JN005766)	Anisakis PB-2009(JN005758)	0,195	0,028
A nascettii(AY260555)	Anisakis PB-2009(JN005758)	0,194	0,028
Anisakis MP-2005(EU718477)	Anisakis PB-2009(JN005758)	0,188	0,027
A physeteris(JN005754)	Anisakis PB-2009(JN005759)	0,230	0,029
A physeteris(AY826721)	Anisakis PB-2009(JN005759)	0,238	0,030
A physeteris(JN005763)	Anisakis PB-2009(JN005759)	0,242	0,031
A brevispiculata(EU624344)	Anisakis PB-2009(JN005759)	0,246	0,034
A paggiae(EU624345)	Anisakis PB-2009(JN005759)	0,225	0,030
A pegreffii(JN005756)	Anisakis PB-2009(JN005759)	0,157	0,023
A pegreffii(EU624343)	Anisakis PB-2009(JN005759)	0,157	0,023
A pegreffii(JN005765)	Anisakis PB-2009(JN005759)	0,165	0,024
A simplex(JN005764)	Anisakis PB-2009(JN005759)	0,162	0,023
A simplex(EU624342)	Anisakis PB-2009(JN005759)	0,162	0,023
A simplex(JN005757)	Anisakis PB-2009(JN005759)	0,164	0,024
A simplexXA pegreffii(JN005768)	Anisakis PB-2009(JN005759)	0,159	0,023
A simplex C(AY826722)	Anisakis PB-2009(JN005759)	0,160	0,024
A ziphidarum(JN005767)	Anisakis PB-2009(JN005759)	0,195	0,029
A ziphidarum(AY826725)	Anisakis PB-2009(JN005759)	0,192	0,028
A ziphidarum(JN005766)	Anisakis PB-2009(JN005759)	0,195	0,028
A nascettii(AY260555)	Anisakis PB-2009(JN005759)	0,194	0,028
Anisakis MP-2005(EU718477)	Anisakis PB-2009(JN005759)	0,188	0,027
Anisakis PB-2009(JN005758)	Anisakis PB-2009(JN005759)	0,003	0,002
A physeteris(JN005754)	Anisakis HC-2005 PB-2010(JN005761)	0,243	0,032
A physeteris(AY826721)	Anisakis HC-2005 PB-2010(JN005761)	0,251	0,033
A physeteris(JN005763)	Anisakis HC-2005 PB-2010(JN005761)	0,255	0,034

A brevispiculata(EU624344)	Anisakis HC-2005 PB-2010(JN005761)	0,218	0,030
A paggiae(EU624345)	Anisakis HC-2005 PB-2010(JN005761)	0,210	0,028
A pegreffii(JN005756)	Anisakis HC-2005 PB-2010(JN005761)	0,262	0,039
A pegreffii(EU624343)	Anisakis HC-2005 PB-2010(JN005761)	0,261	0,038
A pegreffii(JN005765)	Anisakis HC-2005 PB-2010(JN005761)	0,273	0,040
A simplex(JN005764)	Anisakis HC-2005 PB-2010(JN005761)	0,261	0,038
A simplex(EU624342)	Anisakis HC-2005 PB-2010(JN005761)	0,261	0,038
A simplex(JN005757)	Anisakis HC-2005 PB-2010(JN005761)	0,264	0,038
A simplexXA pegreffii(JN005768)	Anisakis HC-2005 PB-2010(JN005761)	0,257	0,038
A simplex C(AY826722)	Anisakis HC-2005 PB-2010(JN005761)	0,258	0,039
A ziphidarum(JN005767)	Anisakis HC-2005 PB-2010(JN005761)	0,234	0,036
A ziphidarum(AY826725)	Anisakis HC-2005 PB-2010(JN005761)	0,230	0,036
A ziphidarum(JN005766)	Anisakis HC-2005 PB-2010(JN005761)	0,230	0,036
A nascettii(AY260555)	Anisakis HC-2005 PB-2010(JN005761)	0,255	0,038
Anisakis MP-2005(EU718477)	Anisakis HC-2005 PB-2010(JN005761)	0,257	0,039
Anisakis PB-2009(JN005758)	Anisakis HC-2005 PB-2010(JN005761)	0,047	0,009
Anisakis PB-2009(JN005759)	Anisakis HC-2005 PB-2010(JN005761)	0,044	0,009
A physeteris(JN005754)	Anisakis HC-2005(EU718474)	0,242	0,031
A physeteris(AY826721)	Anisakis HC-2005(EU718474)	0,250	0,033
A physeteris(JN005763)	Anisakis HC-2005(EU718474)	0,254	0,034
A brevispiculata(EU624344)	Anisakis HC-2005(EU718474)	0,218	0,029
A paggiae(EU624345)	Anisakis HC-2005(EU718474)	0,209	0,028
A pegreffii(JN005756)	Anisakis HC-2005(EU718474)	0,259	0,038
A pegreffii(EU624343)	Anisakis HC-2005(EU718474)	0,258	0,038
A pegreffii(JN005765)	Anisakis HC-2005(EU718474)	0,270	0,039
A simplex(JN005764)	Anisakis HC-2005(EU718474)	0,258	0,037
A simplex(EU624342)	Anisakis HC-2005(EU718474)	0,258	0,037
A simplex(JN005757)	Anisakis HC-2005(EU718474)	0,261	0,038
A simplexXA pegreffii(JN005768)	Anisakis HC-2005(EU718474)	0,254	0,037
A simplex C(AY826722)	Anisakis HC-2005(EU718474)	0,255	0,038
A ziphidarum(JN005767)	Anisakis HC-2005(EU718474)	0,233	0,036
A ziphidarum(AY826725)	Anisakis HC-2005(EU718474)	0,229	0,035
A ziphidarum(JN005766)	Anisakis HC-2005(EU718474)	0,229	0,035
A nascettii(AY260555)	Anisakis HC-2005(EU718474)	0,254	0,038
Anisakis MP-2005(EU718477)	Anisakis HC-2005(EU718474)	0,256	0,038
Anisakis PB-2009(JN005758)	Anisakis HC-2005(EU718474)	0,045	0,009
Anisakis PB-2009(JN005759)	Anisakis HC-2005(EU718474)	0,042	0,008
Anisakis HC-2005 PB-2010(JN005761)	Anisakis HC-2005(EU718474)	0,001	0,001
A physeteris(JN005754)	A typica(JN005760)	0,421	0,055
A physeteris(AY826721)	A typica(JN005760)	0,433	0,057
A physeteris(JN005763)	A typica(JN005760)	0,442	0,058
A brevispiculata(EU624344)	A typica(JN005760)	0,451	0,060
A paggiae(EU624345)	A typica(JN005760)	0,429	0,056
A pegreffii(JN005756)	A typica(JN005760)	0,388	0,050
A pegreffii(EU624343)	A typica(JN005760)	0,389	0,051
A pegreffii(JN005765)	A typica(JN005760)	0,404	0,053
A simplex(JN005764)	A typica(JN005760)	0,384	0,050
A simplex(EU624342)	A typica(JN005760)	0,384	0,050
A simplex(JN005757)	A typica(JN005760)	0,392	0,051
A simplexXA pegreffii(JN005768)	A typica(JN005760)	0,384	0,050
A simplex C(AY826722)	A typica(JN005760)	0,380	0,049
A ziphidarum(JN005767)	A typica(JN005760)	0,313	0,038
A ziphidarum(AY826725)	A typica(JN005760)	0,313	0,038
A ziphidarum(JN005766)	A typica(JN005760)	0,317	0,039

A nascettii(AY260555)	A typica(JN005760)	0,329	0,044
Anisakis MP-2005(EU718477)	A typica(JN005760)	0,327	0,044
Anisakis PB-2009(JN005758)	A typica(JN005760)	0,390	0,054
Anisakis PB-2009(JN005759)	A typica(JN005760)	0,381	0,053
Anisakis HC-2005 PB-2010(JN005761)	A typica(JN005760)	0,374	0,049
Anisakis HC-2005(EU718474)	A typica(JN005760)	0,378	0,050
A physeteris(JN005754)	A typica(EU327688)	0,442	0,058
A physeteris(AY826721)	A typica(EU327688)	0,455	0,061
A physeteris(JN005763)	A typica(EU327688)	0,465	0,061
A brevispiculata(EU624344)	A typica(EU327688)	0,456	0,060
A paggiae(EU624345)	A typica(EU327688)	0,450	0,059
A pegreffii(JN005756)	A typica(EU327688)	0,407	0,053
A pegreffii(EU624343)	A typica(EU327688)	0,408	0,053
A pegreffii(JN005765)	A typica(EU327688)	0,424	0,055
A simplex(JN005764)	A typica(EU327688)	0,402	0,052
A simplex(EU624342)	A typica(EU327688)	0,402	0,052
A simplex(JN005757)	A typica(EU327688)	0,411	0,054
A simplexXA pegreffii(JN005768)	A typica(EU327688)	0,402	0,052
A simplex C(AY826722)	A typica(EU327688)	0,398	0,051
A ziphidarum(JN005767)	A typica(EU327688)	0,327	0,040
A ziphidarum(AY826725)	A typica(EU327688)	0,327	0,040
A ziphidarum(JN005766)	A typica(EU327688)	0,332	0,040
A nascettii(AY260555)	A typica(EU327688)	0,333	0,044
Anisakis MP-2005(EU718477)	A typica(EU327688)	0,343	0,046
Anisakis PB-2009(JN005758)	A typica(EU327688)	0,410	0,057
Anisakis PB-2009(JN005759)	A typica(EU327688)	0,401	0,056
Anisakis HC-2005 PB-2010(JN005761)	A typica(EU327688)	0,389	0,051
Anisakis HC-2005(EU718474)	A typica(EU327688)	0,393	0,052
A typica(JN005760)	A typica(EU327688)	0,001	0,001
A physeteris(JN005754)	A typica(JN005762)	0,421	0,055
A physeteris(AY826721)	A typica(JN005762)	0,433	0,057
A physeteris(JN005763)	A typica(JN005762)	0,442	0,058
A brevispiculata(EU624344)	A typica(JN005762)	0,451	0,060
A paggiae(EU624345)	A typica(JN005762)	0,429	0,056
A pegreffii(JN005756)	A typica(JN005762)	0,388	0,050
A pegreffii(EU624343)	A typica(JN005762)	0,389	0,051
A pegreffii(JN005765)	A typica(JN005762)	0,404	0,053
A simplex(JN005764)	A typica(JN005762)	0,384	0,050
A simplex(EU624342)	A typica(JN005762)	0,384	0,050
A simplex(JN005757)	A typica(JN005762)	0,392	0,051
A simplexXA pegreffii(JN005768)	A typica(JN005762)	0,384	0,050
A simplex C(AY826722)	A typica(JN005762)	0,380	0,049
A ziphidarum(JN005767)	A typica(JN005762)	0,313	0,038
A ziphidarum(AY826725)	A typica(JN005762)	0,313	0,038
A ziphidarum(JN005766)	A typica(JN005762)	0,317	0,039
A nascettii(AY260555)	A typica(JN005762)	0,329	0,044
Anisakis MP-2005(EU718477)	A typica(JN005762)	0,327	0,044
Anisakis PB-2009(JN005758)	A typica(JN005762)	0,390	0,054
Anisakis PB-2009(JN005759)	A typica(JN005762)	0,381	0,053
Anisakis HC-2005 PB-2010(JN005761)	A typica(JN005762)	0,374	0,049
Anisakis HC-2005(EU718474)	A typica(JN005762)	0,378	0,050
A typica(JN005760)	A typica(JN005762)	0,001	0,001
A typica(EU327688)	A typica(JN005762)	0,002	0,002