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XXIV ciclo

**PATTERNS OF GENETIC VARIATION OF A
MONOGENOIDEAN LESSEPSIAN PARASITE**

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Abstract

After the opening of the Suez Canal in 1869 and after the further changes of the water regime of the canal and the near area of the Eastern Mediterranean Sea, the biogeographical barrier existing until that moment, between the Red Sea and the Mediterranean Sea, has been, as time goes by, weakened, allowing the contact between two different biota that was separated for 12 Million years.

This work is focused on the mechanisms that set the spread and the colonization of Mediterranean Sea from *Glyphidohaptor plectocirra*, monogenoidean parasite of rabbit fish *Siganus rivulatus* (Siganidae), checking also the relationship between the spread of the host species, known to bibliography, and of the parasite, experimentally analysed. Based on available literature, several genetic studies of Lessepsian species often demonstrate the absence of a genetic bottleneck in a wide plethora of taxa, from plants to fish, but information regarding the genetic responses of their parasites in the newly colonized ecosystems is still lacking.

In detail, in this work, the genetic flow relationships between five populations was estimated, three from the Red Sea (Nabq and Ismalia, Egypt, and Eilat, Israel) and two from Mediterranean Sea (Rhodes Island, Greece and Tel Aviv, Israel) by sequencing a portion of the mitochondrial CoxI gene.

G. plectocirra specimens were extracted from gill arches of *S. rivulatus* and morphologically identified by analysing haptor sclerotized structures. After the morphological identification of specimens, two DNA regions were amplified, one mitochondrial, about 688 bps long, coding for the Cytochrome Oxidase I (COI) and one nuclear, 681 bps long, coding for the terminal region of the 18S gene, that include the entire ITS1 and a portion of the 5.8S gene, was then amplified using primers S1. The 681 bp alignment of 60 rDNA sequences found in all populations showed only two polymorphic sites, which distinguished only three haplotypes (data not shown). Thus, the low variability of this marker at the species level in the monogenoidean parasites was confirmed, and any downstream analysis was performed only on the CoxI gene.

For CoxI marker about 197 sequences were obtained for a total of 58 haplotypes, showing 65 polymorphic sites (27 parsimony informative), and no gaps were observed. In the original populations were identified more than 30 haplotypes, half of which was private; while in the Mediterranean population were identified about 20 haplotypes, just under half of which was private. In general the values of haplotype and nucleotide diversity were higher within the origin population. The maximum parsimony network obtained showed absence of geographic structure, and is characterized by a star shape: by an ancestral haplotype are derived a large number of derived haplotypes, which are very similar to each other. Indeed, despite the large number of variants, derived haplotypes differ from the central one by a low number of mutations and, with very few exceptions, are equally low represented; in most of the cases derived haplotypes were identified in just one individual.

The analysis of molecular variance showed that the Mediterranean populations may be considered a subgroup with reduced variability but that the genetic variance is attributable mostly to differences within individual populations. The maximum parsimony network, with star shape, is characterized

by one central haplotype from which all the others derive. The analysis of molecular variance confirm that the genetic variance is attributable for 97% to differences within the populations, supporting that the Mediterranean populations are a subgroup of Red Sea, even if they show some private haplotypes.

An analysis of Bayesian inference showed a significant unidirectional genetic flow, from Red Sea to Mediterranean Sea. While it even showed a large genetic dimension of Mediterranean populations that confirms the presence of different colonization source, not sampled, in Red Sea.

Despite evidence of a slight decrease in the genetic diversity of Mediterranean populations, a simulation analysis based on coalescent theory demonstrated the absence of significant bottlenecks, but there was directional selection along a cline moving further from the Suez Canal. The absence of bottlenecks was congruent with that described for *G. plectocirra* hosts *Siganus rivulatus* and *Siganus luridus*, and reflected a common history of high propagule pressure during initial colonization, and constant or repeated gene flow from the Red Sea to the Mediterranean area. However, directional selection

was peculiar to the parasites and likely originated from parasite genotype \times environment interactions. Finally, an anisotropic contribution of Red Sea populations to the Lessepsian invasion was demonstrated.

Keywords: Lessepsian, invasion, COI, Siganiidae, *Siganus* Monogenoidea, *Glyphidohaptor*, directional selection, bottleneck

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1. INTRODUCTION

1.1 Monogenoidean parasites

Monogenoids are one of the largest groups of ectoparasitic flatworms, belonging to Platyhelminthes, and they are divided in two orders: Monopisthocotylea and Polyopisthocotylea, on the basis of the opisthaptor morphology (the organ able to attach the host). The vast majority of monogenoidean species infects gills, skin and fins of freshwater and marine fishes, while other ones adopt an endoparasitic life's style in the mouth cavity and urinary bladder of turtles and amphibians. They are rarely longer than about 2 cm but a few species infecting certain marine fish are larger. Monogenoids lack respiratory, skeletal and circulatory systems. Monogenoids attach mechanically to hosts using several specialized structures such as anchors, hooks and clamps, located in the posterior region of the body (foot). They also attached chemically to the host through the adhesive proteins, secreted by the cephalic glands located in the anterior region of the body. Like other flatworms, Monogenoids have no true body cavity (coelom) and they have a simple digestive system consisting of a mouth opening with a muscular pharynx and an intestine without terminal opening. Generally, they also are hermaphroditic with functional reproductive organs of both

sexes occurring in one individual. They have a head region that contains concentrated sense organs and nervous tissue (Bychowsky, 1961).

Monogenoids have a monogenic lifecycle (they require a single host to complete a whole lifecycle). Monogeneans possess the simplest life cycle among the parasitic platyhelminths. Although they are hermaphrodites, the male reproductive system becomes functional before the female part. The eggs hatch releasing a heavily ciliated larval stage known as an *oncomiracidium*. The *oncomiracidium* has numerous posterior hooks and is generally the life stage responsible for transmission from host to host.

1.2 Spinefoots and their parasites (species of interest)

Spinefoots (or rabbitfishes), *Siganus* spp. (Siganidae), are marine perciform fishes, originally occurring in the tropical and subtropical Indo-Pacific Region, with the exception of the waters of the Hawaiian Islands and Easter Island (Woodland, 1990).

Only few species of spinefoots, 8 of the 28 valid species listed by Froese and Pauly (2011), have been reported to be hosts for dactylogyrid parasites (Monogenoidea). Three other species, comprising the streamlined spinefoot, *S. rostratus* (Valenciennes) (now *S. argenteus* (Quoy and Gaimard)), the foxface, *S. vulpinus* (Schlegel and Müller), and the streaked spinefoot, *S. javus* (Linnaeus), have been examined in previous studies, but dactylogyrids have not been recorded from these hosts (Paperna, 1972; Lim, 2002). For what concern monogenoidean parasites, four dactylogyrid genera, *Tetrancistrum* Goto and Kikuchi, 1917, *Pseudohaliotrema* Yamaguti, 1953, *Pseudohaliotrematoides* Yamaguti, 1953, *Glyphidohaptor* Ktitsky et al, 2007, contain species that are actually known to infest spinefoots.

The present population genetic study is only focused on the monogenoidean parasite *Glyphidohaptor plectocirra* (Paperna, 1972), infesting the marbled spinefoot *Siganus rivulatus* Forsskål and Niebuhr, 1775.

Siganus rivulatus (Fig. 1.1 and 1.2)

Class Actinopterygii

Order Perciformes

Suborder Acanthuroidei

Family Siganidae

Marbled spinefoot, *Siganus rivulatus* Forsskål and Niebuhr, 1775, inhabits shallow waters and generally in schools of 50 to several hundred individuals and prefers protected areas. He is an euryhaline fish and feeds by grazing on algae. It is originally distributed in the eastern portion of the Indo-Pacific region (Woodland 1983) (Fig.1.3).

The main morphological features are as follow: 13 dorsal spines, 10 dorsal soft rays, 7 anal spines, 9 anal soft rays, 23 vertebrae. Upper body is gray, green or brownish, silvery below; iris is iridescent silver or golden. Body color patterns extend to the fins. Spines are slender, pungent and venomous. Other peculiar features are preopercular angle of 88°-96°, cheeks scaled and midline of thorax, isthmus and midline of belly without scales. Frightened fish become mottled or with 6 diagonal zones across side. Tip of broad-based flap of anterior nostril reaching at least halfway to orifice of posterior nostril (Froese and Pauly 2011 and Duray 1998).

S. rivulatus is one of the most successful Lessepsian migrants and it was one of the first fish species to settle in the eastern Mediterranean coasts after the opening of the Suez Canal in 1869 (Por 1978). The first record of the *S. rivulatus* in the Mediterranean Sea dates back to 1927 in the eastern Mediterranean (Tortonese 1970); it has been followed by the strictly related conspecific species *Siganus luridus* in 1956 (Azzurro et al 2006). In the eastern Mediterranean region *S. rivulatus* is actually going to become a fish species of commercial interest, particularly for the Greek market (Papaconstantinou 1990).

Reproductive season of *S. rivulatus* is comprised between May and August both in Mediterranean and Red Sea (Popper, 1979). However, Popper & Gundermann (1975) comparing Red Sea populations with Mediterranean ones, observed a reduction in the duration of the reproductive season in the new colonized area. Indeed, previous studies, focused on siganids reproductive biology, demonstrated as water temperature can affect the occurrence of the reproductive season in terms of duration and period of the year. In the eastern area of the Mediterranean basin,

water temperature is higher in summer and colder in winter than in the Red Sea and, due to *S. rivulatus* reproductive temperature range (from 24° C to 29° C), the effective reproductive period in the Mediterranean basin has to be reduced of about one month, in order to face the climatic difference (Lam & Soh, 1975; Popper et al 1976; Amin 1985).

Growth rate of the marbled spinefoot is determinate by several biotic and abiotic factors (Brett & Groves 1979), including water temperature (Fry 1971; Somero, Dahlhoff & Lin 1996). Water temperature influences several physiological processes, such as food consumption, metabolic rate and reproduction (Jobling 1994 and Hillman et al 1999), and, consequently, the growth rate of the fishes (Brett 1979 and Jobling 1996). *S. rivulatus* grows rapidly between May and January (with temperature above 17° C) and growth rate significantly decreases when water temperature is lower. Growth does not seem to be influenced from water temperature hot spots during the summer period, differently from reproduction that is highly associated to such events. *S. rivulatus* average age is six years but it can reach also 8 years and *sex ratio* is approximately

1:1, independently from age and growth rate (Bariche et al, 2003).



Figure 1.1 – Marbled spinefoots, *Siganus rivulatus*.



Figure 1.2 – Marbled spinefoots, *Siganus rivulatus*. Pictures by Massimo Boyer.

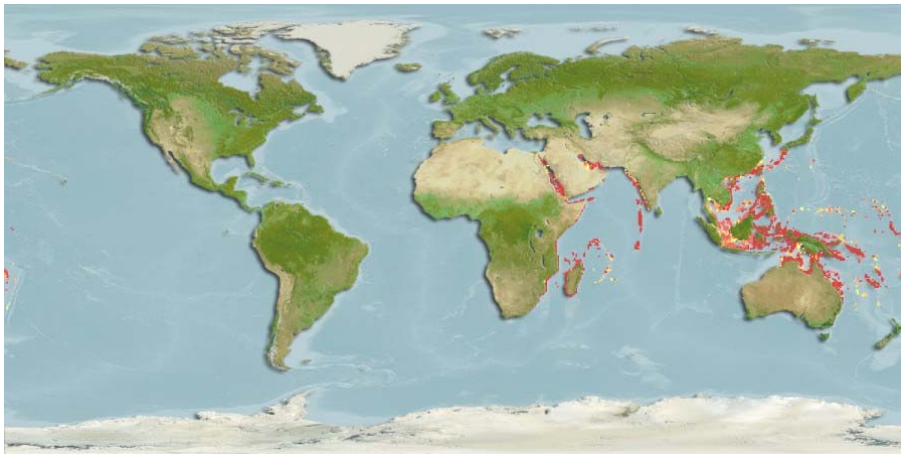


Figure 1.3 – Original Worldwide distribution of marbled spinefoots, *Siganus rivulatus*. (www.fishbase.com).

In the last years, the most part of research activity about marbled spinefoot was focused on the identification of the features of this species that allowed his extremely successful invasion of the Mediterranean Sea, in order to better understand the processes of colonization and to recognize future potential invasive species and threatened ecosystems. Some authors stated that success of invasions is generally determinate by intrinsic features of invasive populations, such as genetic variability, growth rate, capability to expand trophic niches, reproductive strategies, and tolerance to abiotic stresses (Elrich, 1989; Byers and Goldwasser, 2001). An invasive colonization scenario should be genetically characterized by the “founder effect” or “bottleneck”. The genetic structure of an invasive population is dependent from several factors such as effective genetic population size, origin of different invaders, genetic diversity of source populations, and potential presence of genetic structure in the source population (Van Driesche and Van Driesche 2000). For what concern marbled spinefoot colonization process, it has been demonstrated by Azzurro et al (2006) that Mediterranean populations showed a reduced genetic variability compared to Red Sea population and that Mediterranean haplotypes were a subsample of Red Sea haplotypes pool. The simplest explanation

for the variability reduction in the Mediterranean population compared to Red Sea population has to be traced back to the difficult migration through the Suez Canal that create a weak biogeographic barrier (filter), reducing the genetic flow from the Red Sea to the Mediterranean basin. However, some studies demonstrated that in some cases the genetic diversity of the Mediterranean populations can be extremely high and, in such situations, there is no evidence of founder effect or bottleneck (Bonhomme et al, 2003 e Hassan et al, 2003). For a more exhaustive treatment of such aspects, please refer to the following section “1.5 Lessepsian invasions” and chapter “4 - Discussion”.

Glyphidohaptor plectocirra

Class Monogenoidea

Subclass Polyonchoinea

Order Dactylogyridea

Family Dactylogyridae

Glyphidohaptor plectocirra

This study is focused on the monogenoidean flatworm *Glyphidohaptor plectocirra* (Fig. 1.4 and 1.5), a parasite belonging to Dactylogyridae family, infecting gills of marine fishes belonging to Siganidae family, including marbled spinefoot, *Siganus rivulatus*.

The specific identification of *G. plectocirra* can be based on the morphology of several key structures such as haptor sclerites (transverse bar and anchors) and copulatory sclerites (Fig. 1.4).

Monogenoidean parasite *G. plectocirra* is characterized by the following morphological features.

- Body: Fusiform, slightly flattened dorsoventrally, composed of the body proper (cephalic region, trunk, short peduncle), haptor. Tegument smooth.
- Cephalic region: Two terminal, 2 bilateral cephalic lobes, 3 pairs of bilateral head organs; cephalic glands posterolateral to pharynx. Eyespots absent; chromatic granules minute, subovate, scattered throughout cephalic region and anterior trunk.
- Gastrointestinal tract: Mouth subterminal, midventral, at level of head organs, opening into buccal tube; buccal tube extending posteriorly along body midline to pharynx; pharynx made up of muscular, glandular bulb; esophagus short to moderately long; intestinal ceca 2, confluent posterior to gonads, lacking diverticula.
- Reproductive system: Common genital pore midventral, immediately posterior to intestinal bifurcation. Gonads intercecal; germarium lying to

right of anterior portion of testis. Proximal portion of vas deferens not observed; seminal vesicle a simple dilation of vas deferens immediately posterior to base of male copulatory organ (MCO) (Fig. 1.4); bilateral prostatic glands dorsolateral to copulatory complex; 2 elongate U-shaped prostatic reservoirs emptying individually into base of MCO. Copulatory complex composed of basally articulated MCO, accessory piece. MCO tubular, with basal flange, enclosed in variably developed sheath. Accessory piece rod shaped with platelike projection arising along proximal half of rod. Oviduct short, receiving bilateral ducts of vitellarium, vagina; ootype, seminal receptacle not observed; uterus extending anteriorly along body midline to common genital pore. Vaginal pore dextromarginal, in anterior trunk; vagina with distal vestibule, variably coiled or meandering tube extending to oviduct. Vitellarium coextensive with gut, absent in regions of other reproductive organs.

- Haptor (Fig. 1.4): bilobed on posterior margin, subtrapezoidal, with truncate or slightly indented

posterior margin wider than anterior margin, armed with dorsal, ventral anchor-bar complexes, 7 pairs of similar hooks with ancyrocephaline distribution (Mizelle, 1936; Mizelle and Price, 1963). Dorsal, ventral anchors similar; each with large broad base with subequal longitudinally striated roots, short shaft, short rapidly tapered open point. Ventral bar with subterminal globose expansions from each of which anterolaterally directed lobulate projection arises; dorsal bar flat, ribbon like, delicate (often difficult to observe), with ends directed anteriorly approximately perpendicular to median portion of bar. Hook with protruding thumb, slender shank composed of 1 subunit; filamentous hook (FH) loop about shank length (Kritsky et al 2007).

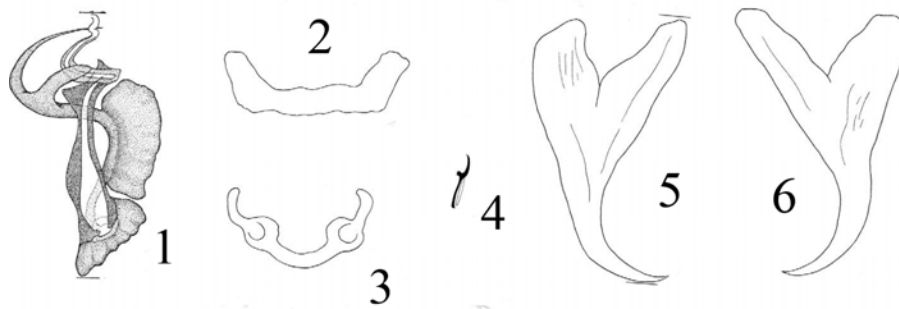


Figure 1.4 – Haptoral and copulatory sclerites of *Glyphidohaptor plectocirra* (Paperna, 1972), representing the key structures for the morphological identification of this parasite species. (1) Copulatory complex (ventral). (2) Dorsal bar. (3) Ventral bar. (4) Hook. (5) Ventral anchor. (6) Dorsal anchor. Drawing modified from Kritsky et al, 2007.

Only few molecular studies have been performed on monogenoidean parasites, and most of them aim to describe evolutionary patterns, specific identification or coevolutionary scenarios (see for example Desdevises et al 2002, Plaisance et al 2008, Kritsky et al 2010, Salogni et al 2008, Aquaro et al 2011.) more than phylogeographic or genetic population dynamics. Nevertheless, monogenoidean parasite ecology, including host - parasite relationships, both in natural and laboratory conditions has been largely studied (see for example Littlewood et al 1997, Simkova et al 2000, 2001a, 2001b; King and Cable 2007, King et al 2009, Strona 2010.). Despite of the presence of such ecological studies, large scale genetic analyses focused on

ecological processes are still few for monogenoidean and for the most part of parasitic *taxa*. In addition, only very few studies of population genetic are focused on parasite as invasive organisms and none is focused on a Lessepsian migrant parasite, such as *G. plectocirra*, which is the target of this work (please refer to the following section “1.5 Lessepsian invasions” and chapter “4 - Discussion” for further details about biological invasion and Lessepsian migration).

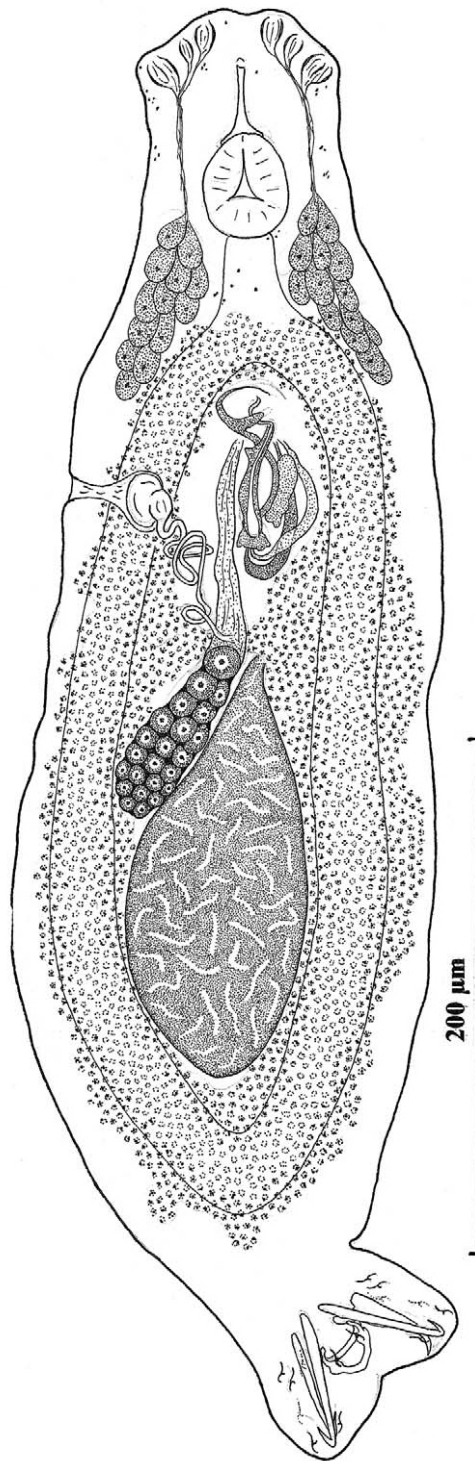


Figure 1.5 – Whole-mount drawings of *Glyphidohaptor plectocirra* (Paperna, 1972). Drawing modified from Kritsky et al, 2007.

1.3 Definition of “Lessepsian Migration”

The term “Lesspsian Migration” was coined when it became evident that the migration through the Suez Canal towards Mediterranean Sea is a unique phenomenon in modern biogeography (Por, 1969 and 1971). Actually, Lessepsian migration appears to be mainly unidirectional, from the Red Sea to the Mediterranean Sea. Moreover, the specific term “Lessepsian Migration” does not include migrational events which occurred by passive transports to harbours far from the Canal and does not included isolated events of opposite migration, from the Mediterranean Sea to the Red Sea (Por, 1978).

1.4 Historical background

About 12 million years ago, the worldwide circumtropical marine connection was interrupted. This event was the baseline for all the marine zoogeographical processes up to now. In particular, the Isthmus of Suez, separating eastern Mediterranean from Indo-Pacific region, probably appeared in the upper Miocene (approximately from 11.000 to 5.000 million years ago)

(Picard, 1943 and Swartz and Arden, 1960), this was due to a tectonic upheaval. Nile sediments contributed to the closure of the Isthmus (Moshkovitz, 1968); following the Upper Miocene upheaval of the Ethiopian Highlands, the Nile, until then a relatively small river, started to increment the transported amounts of silt suspensions which resulted in the interruption of the contact between the Paleomediterranean and the Gulf of Suez branch. Moreover, both the Mediterranean and the Red Sea encountered hypersaline to brackish periods during the Upper Miocene, which likely exterminated the pre-existing fauna. During the Pliocene, the Paleomediterranean was repopulated with tropical West African species, while the Red Sea was subject to a colonization of Indo-Pacific species. Consequently, the “totally” dissimilar fauna of the two seas, before the opening of the Suez Canal in 1869, has its origins on a complete separation of the two basins, involving a separated evolution of the two biotas, since the Miocene.

In 1869, the opening of the Suez Canal (Fig. 1.6) created the first salt-water passage between the Mediterranean Sea and Red Sea. The Red Sea is higher than the Eastern Mediterranean

Sea, so the canal works as a tidal strait that transfers Red Sea water into the Mediterranean Sea.

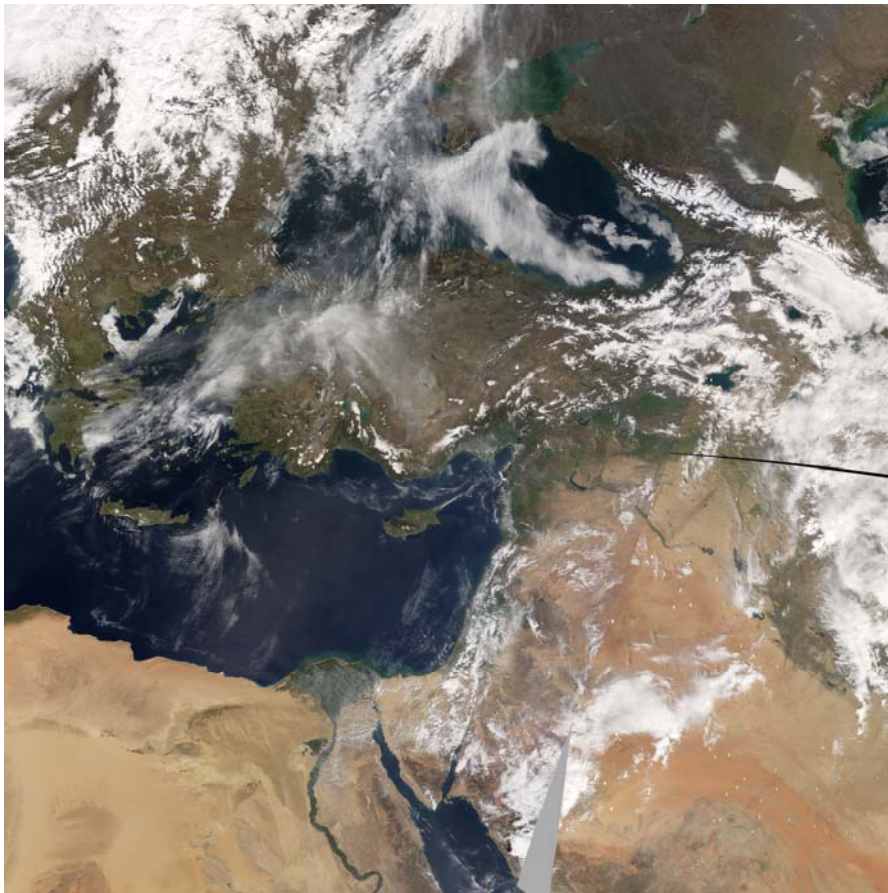


Fig. 1.6 – Satellite view of Eastern Mediterranean and Northern Red Sea Regions.

The Bitter Lakes, hypersaline natural lakes forming part of the canal (Fig. 1.7), blocked the migration of Red Sea species

towards the Mediterranean for many years, even if, as the salinity of the Bitter Lakes gradually equalized with that of the Red Sea, the barrier to migration through the Suez Canal was removed, and the biota from the Red Sea started to colonize the Eastern Mediterranean Sea. In the 1960s, the construction of the Aswan High Dam on the Nile River decreased the inflow of freshwater and nutrient-rich suspended solids from the Nile into the eastern Mediterranean Sea, changing conditions in the eastern Mediterranean that become even more similar to the Red Sea. These events increased the impact of the existing invasive processes and facilitated the occurrence of new ones (Dov Poor, 1978).

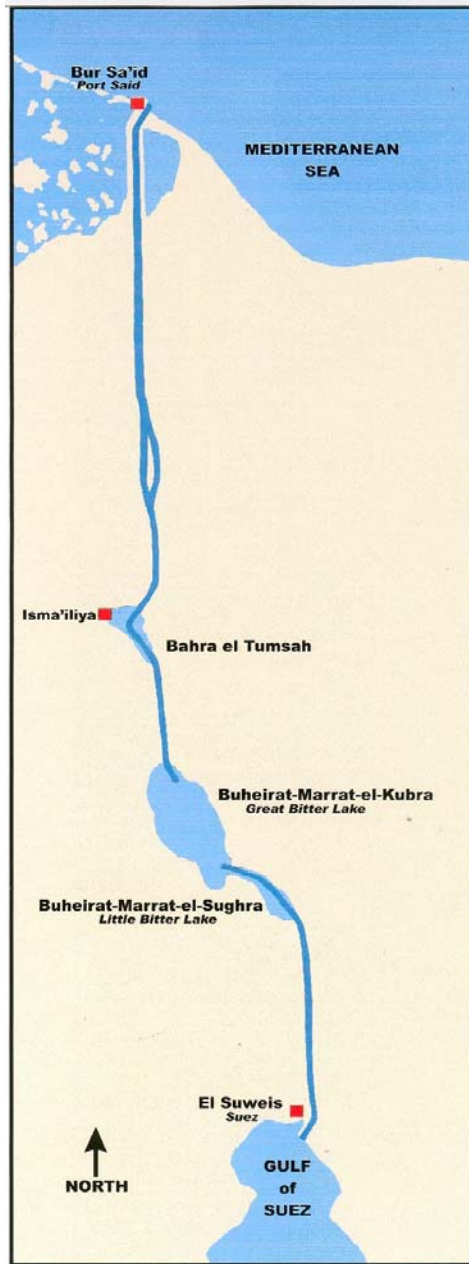


Fig. 1.7 – Actual Geography of the Suez Canal.

1.5 Lessepsian invasions

Biological invasions have an increasing impact on ecological and economic balance and the magnitude of this threat is increasing globally (Convention on the Conservation of European Wildlife and Natural Habitats 2002; Walpole et al 2009). Invasive alien species can affect ecosystem processes, and decrease native species abundance and richness via competition, predation, and hybridization, while their indirect effects can change community structure and affect genetic diversity (see McGeoch et al 2010 for review). At the same time these species offer new opportunities to explore ecological and evolutionary processes (Bernardi et al 2009).

The Lessepsian invasions (Por 1978) initiated after the opening of the Suez Canal in 1869 constitute a unique case, where the timing of invasion, the invasion route, and the invader's geographic source are known. Lessepsian migrants include a variety of taxa (Fig. 1.8) distributed over at least 300 new species, including 71 fishes (Golani and Appelbaum-Golani 2010). Genetic studies of the Lessepsian invasion have mainly focused on fishes (Azzurro et al 2006; Bariche and Saad 2004;

Bonhomme et al 2003; Bucciarelli et al 2002; Golani and Ritte 1999; Golani et al 2007; Hassan et al 2003; Hassan and Bonhomme 2005) and molluscs (Lavee and Ritte 1994; Lavie and Nevo 1986; Sirna Terranova et al 2006). Other invertebrates (Iannotta et al 2007; Karako et al 2002; Lai et al 2008) and plants (Procaccini et al 1999) have been considered in a few cases, but Lessepsian parasites have rarely been considered. This lack of knowledge reflects an historical scientific disregard for this group of species. Considering that a rich parasite fauna is generally carried by wild animals, parasite species are likely to have been transported from donor regions by non-indigenous hosts and this is clearly underestimated. According to Zenetos et al (2008), 17 species of parasites (6 Monogenoidea, 5 Crustacea, 5 Protozoa and 1 Digenea) have been recognized as Lessepsian, and newly described species are continuously added to the list (El-Rashidy and Boxshall, 2011). The first documented case of a monogenoidean invading a new biogeographical region by 'natural' extension of its host range was that of the gill ectoparasite *Polylabris* cf. *mamaevi* infecting *Siganus rivulatus* (Pasternak et al 2007).

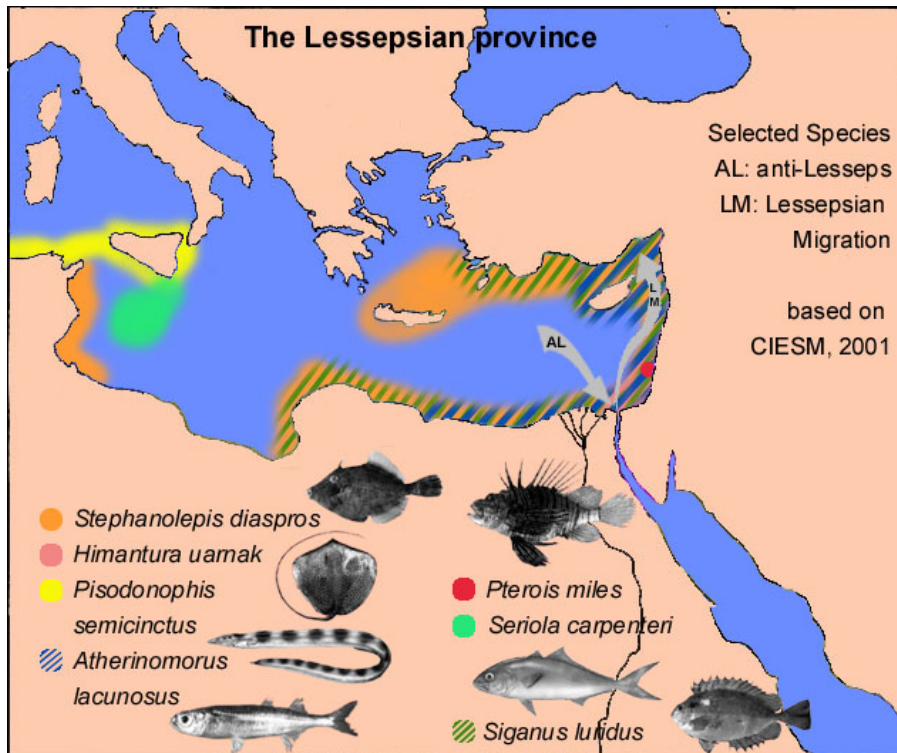


Fig. 1.8 - The Lessepsian Province of the Mediterranean featuring selected species (figure by Madl, 2001).

Invasive species generally harbor a subset of their original genetic pool and in many cases reduced genetic variation can be expected (Allendorf and Lundquist 2003) to have negative effects on their capability to adapt to environmental conditions (Taylor and Hastings 2005). Arguments including genetic

adaptation, epigenetic adaptations, or simple genetic drift have been often invoked to explain the success or failure of bio-invasive events (Roman and Darling 2007; Pérez et al 2006; Lee 2002; Tsutsui et al 2000). Nevertheless, genetic studies have often failed to demonstrate a link between genetic diversity and invasive success (Roman and Darling 2007).

In the case of Lessepsian invasions, (*e.g.*, Sax and Brown 2000), the establishment of invaders in the Mediterranean should be reflected in genetic bottlenecks, because they are a subsample of the original populations. Nevertheless, existing information only highlighted founder effects in two of 12 species studied (Bernardi et al 2009).

Surprisingly, genetic adaptation has never been detected among the studied species, despite the great environmental changes invasive populations face when colonizing the colder Mediterranean waters. These patterns appear transversely recurrent for different ecological or trophic systematic features of the studied species, whereas parasites may provide unexpected departures because of the many different interactions

between host genotype, parasite genotype, and environment during host dispersal (Wolinska and King, 2009).

Alien parasites are a peculiar kind of biological invader, because their populations are structured by the dynamics and movements of their hosts (Blouin et al 1995; McCoy et al 2003; Criscione and Blouin, 2004). Very few published studies have compared the genetic structure and diversity of both the invasive hosts and their parasites. In some cases the genetic structure of parasites can be more evident than, or evident as, that of their hosts (Criscione et al 2005), or depend strictly on the dispersal of their hosts, as in the case of the Asian mud snail *Batillaria attramentaria* and two digenean trematode parasites (Miura et al 2006). The genetic structure and diversity of the trematode HL6, which has a direct life cycle, strictly reflects that of its host. In contrast, the genetic diversity of the trematode HL1, which uses sea birds as intermediate hosts, was much higher and this was probably because of the increased propagule pressure imposed by bird migrations. If this pattern is typical, then one might expect that parasite population structure would be very rapidly established within a few generations of a successful invasion,

given particular barriers to gene flow or differential selection regimes (Wielgoss et al 2008).

In the current study, we inferred the genetic structure of a Lessepsian parasite, *Glyphidohaptor plectocirra*, which parasitizes *Siganus luridus* and *S. rivulatus* both in the Red Sea and in the Mediterranean Sea (Diamant, 1989; Galli et al 2007). The introduction of the Monogenoidea *G. plectocirra* (Kritsky et al 2007) (Synonyms: *Pseudohaliotrema plectocirra* Paperna 1972; *Tetrancistrum plectocirra* Lim 2002) was first described by Paperna (1972) in the northwestern Gulf of Eilat near Taba and Coral beach (Red Sea), and in the Gulf of Suez near Ras abu-Rudeis (Red Sea).

S. rivulatus was first recorded in the 1920s (Steinitz, 1927) and it was one of the first Lessepsian migrants to be found in the Mediterranean. Breeding populations have been established for a long time and it is currently one of the most abundant species in the Levantine rocky shore area, where it makes considerable contribution to local artisanal fisheries (Bonhomme et al 2003). *S. luridus* was first recorded in the Mediterranean in 1955 (Ben-Tuvia 1964) and expanded soon

afterwards. Recent genetic studies based on both mitochondrial and nuclear markers (Azzurro et al 2006; Bonhomme et al 2003; Hassan et al 2003) have highlighted high gene flows from the Red Sea to Mediterranean populations, with no evidence of true bottlenecks. Azzurro et al (2006) actually detected a lower genetic divergence in the Mediterranean population of *S. luridus* along the east-west direction of the expanding colonization. Thus, it was suggested that the continued migration of individuals through the Suez Canal had deleted specific signatures of early colonization events. The goal of our study was to explore the genetic structure of, *G. plectocirra* by estimating: 1) genetic variability within donor and invasive populations; 2) patterns of gene flow and dispersal; 3) differences or similarities with existing information regarding the genetics of invasion by its hosts, *S. luridus* and *S. rivulatus*.

2. MATERIALS AND METHODS

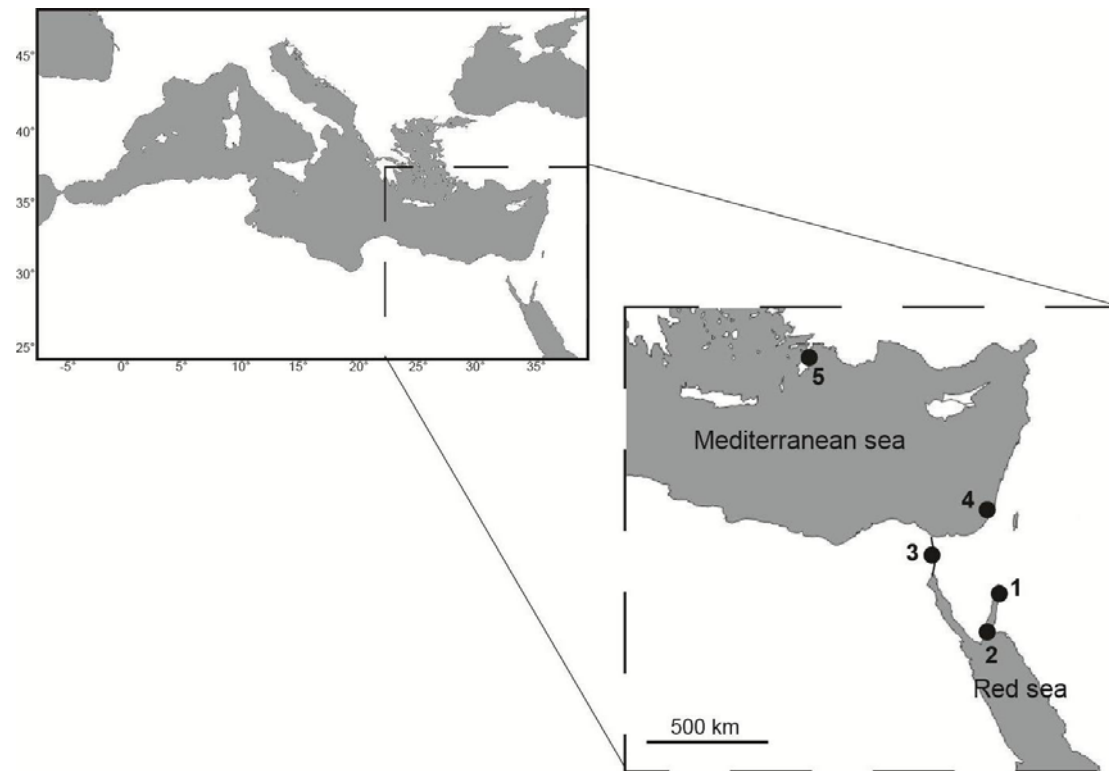
6.1 Area of interest

The present work is based on samples and data collected from five populations (Fig. 2.1) of marbled spinefoot, *Siganus rivulatus*, hosting monogenoidean parasite *Glyphidohaptor plectocirra*. Marbled spinefoot populations belong to five different localities of the northern Red Sea, comprising Suez Canal, and eastern Mediterranean Sea, as summarized in the table below (Table 2.1).

Basin	Country	Population	Geographic coordinate
Red sea	Israel	Eilat	29° 33' 30" N 34° 57' 32" E
	Egypt	Nabq Managed Resources Protected Area – South Sinai	28° 02' 52" N 34° 26' 21" E
		Suez Canal - Ismailia	30° 04' 00" N 31° 16' 00" E
	Mediterranean Sea	Greece	Rhodes Island
Israel		Tel Aviv	32° 4' 0" N 34° 46' 0" E

Table 2.1 – Selected populations of marbled spinefoot, *Siganus rivulatus*, hosting monogenoidean parasite *Glyphidohaptor plectocirra*.

Fig. 2.1 - Geographical location of the five populations of *Glyphidohaptor plectocirra* in the Mediterranean and Red seas. 1 = Eilat, Israel; 2 = Nabq, Egypt; 3 = Ismailia, Egypt; 4 = Tel Aviv, Israel; 5 = Rhodes, Greece.



2.2 DNA amplification

A total of 197 specimen of *G. plectocirra* were collected from host gill lamellae using dissecting needles under a stereomicroscope. Before parasite collection, the gills of each fish host were preserved in DMSO-NaCl solution (20% DMSO, 0.25 M disodium EDTA, and NaCl to saturation, pH 7.5), according to Seutin et al (1991), Strona et al (2009) and Kritsky et al (2010). At the laboratory, parasite specimens were extracted from the gills with a small probe, the haptor region was excised from the body of the parasite, washed with distilled water and put on a slide in a digestion solution drop (25 µl distilled water, 2.5 µl of 10X digestion buffer -10mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% SDS- and proteinase K -final concentration of 100 µg/ml-); the slides were then incubated at 50° C and monitored regularly from 5 to 15 minutes until the lysis occurred. After digestion the proteinase was inactivated by heating slide at 94° C for 10 minutes (Harris et al, 1999). Digested haptors were directly observed for morphological identification of the specimens (Aquaro et al 2009 and Salogni et al 2008). After identification, preserved body regions of the specimens were

washed several times in distilled water for removal of the salts contained in the DMSO solution.

After morphological identification, a DNA extraction protocol modified from Zietara et al (2000) was applied on the bodies of the parasites. In detail, samples (parasites bodies) were dropped into 10 µl of 1× PCR buffer solution and, subsequently, incubated for 10 min at 90 °C in order to deactivate DNAses. Then, 1 µl of K Proteinase was added to the solution and samples were incubated at 55 °C for 30 min. A subsequent incubation at 90 °C for 10 min was used to deactivate K proteinase.

A portion of about 900 bp of the mitochondrial CoxI gene was then amplified by PCR using the primers COI_Mono_5 and COI_Mono_int3 primers, according to an amplification protocol of Plaisance et al (2008), partially modified in order to adapt it on the target species, improving his efficiency. In detail, the amplification was carried out using the following primers COI_mono_5 and COI_mono_int3 (Plaisance et al, 2008). PCR were conducted with a single extension in a 50µl solution containing 1.5 U Taq polymerase, 10 mM Tris-HCl, 50 mM

KCl, 1.5 mM MgCl₂, 200 μM of each dNTP, 10pM of each primer and 2 μl of undiluted DNA solution. Thermal cycling was performed with an initial denaturation for 3 min at 94° C, followed by 40 cycles (30 sec at 94° C, 30 sec at 45° C and 2 min at 72° C), with a final extension of 7 min at 72° C¹⁹). The amplified products were run on a 1.5% agarose gel containing ethidium bromide. Sequencing was performed with COI_mono_int3.

For a subset of samples, a portion of about 1900 bps of the 18S gene was then amplified by PCR using two primers WormA and WormB (Littlewood & Olson, 2001) according to the protocol modified from Plaisance L. et al (2004). In detail, PCR were conducted in a 25 μl solution containing 0.8 U Taq polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 μM each dNTP, 10 pM of each primer and 2 μl of undiluted DNA solution. The thermal cycle comprises a initial denaturation phase at 94°c for 3 min, followed by 40 cycles including : i) a denaturation at 94°C for 30 s; ii) an annealing at 56°C for 30 s; iii) an extension at 72°C for 2 min. Finally, an extension of 10 min. at 72°C was set followed by 10 min. at 10° C. Moreover, due to some problem in the above mentioned PCR

amplification, the terminal region of the 18S gene of the same subset of samples, that include the entire ITS1 and a portion of the 5.8S gene, was then amplified using primers S1 (Sinnappah et al 2001) and IR8 primers (Šimková et al 2003). In detail, PCR were conducted in in a 20 µl solution containing 1 U Taq polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 µM each dNTP, 1 µM of each primer and 2 µl of undiluted DNA solution. The thermal cycle comprises a initial denaturation phase at 95°C for 4 min, followed by 38 cycles including : i) a denaturation at 95°C for 1 min; ii) an annealing at 57°C for 1 min; iii) an extension at 72°C for 1 min and 30 s. Finally, an extension of 10 min. at 72°C was set followed by 10 min. at 10° C.

Amplified products were run on a 1.5% agarose gel containing ethidium bromide. When multiple amplified bands were detected, the specific products were excised from the gel and purified using Perfectprep Gel Cleanup kits (Eppendorf). Finally, the amplified products were sequenced in both directions (with both IR8 and S1 primers).

Sequences were finally aligned using BioEdit 5.0.9 (Hall 1999). Identification of polymorphic and parsimony-informative sites was conducted using DNASP 5.00 software (Librado and Rozas 2009).

2.3 Phylogenetic and demographic analysis

A Minimum Spanning Network (MSN) among haplotypes, embedding all Minimum Spanning Trees, was built using the software Arlequin 3.5 (Excoffier and Smouse 1994; Excoffier and Lischer 2010).

Levels of genetic diversity within and among populations were tested by hierarchical analysis of molecular variance (AMOVA; Excoffier et al 1992) using Arlequin 3.5 (Excoffier and Lischer 2010) with groups set as the Mediterranean vs. Red sea populations (including the Ismailia population). Potential isolation among populations was tested by estimating the pairwise fixation indices F_{st} and testing the significance by haplotype permutations among populations (1000 replicates), as implemented in Arlequin 3.5.

Hypothesis testing evaluated the amount of potential bottlenecks experienced by *G. plectocirra* populations invading the Mediterranean Sea. At this regard, estimated values of haplotype diversity (For. 2.1) (H), nucleotide diversity (π) (For. 2.2) (Nei 1987), and Tajima's D (For. 2.3) (Tajima 1989a) test of selective neutrality were computed for all populations, using Arlequin 3.5.

$$\hat{H} = \frac{n}{n-1} \left(1 - \sum_{i=1}^k p_i^2 \right)$$

Formula 2.1 – Haplotype diversity (H), where p_i is the relative haplotype frequency of each haplotype in the sample and n is the sample size, as defined by Nei, 1987.

$$\hat{\pi} = \sum_{i=1}^k \sum_{j<i} p_i p_j \hat{d}_{ij}$$

Formula 2.2 – Nucleotide diversity (π), where p_i and p_j are the respective frequencies of the i^{th} and j^{th} sequences and d_{ij} is the number of nucleotide differences per nucleotide site between the i^{th} and j^{th} sequences, as defined by Nei, 1987.

$$D = \frac{\hat{\theta}_{\pi} - \hat{\theta}_S}{\sqrt{Var(\hat{\theta}_{\pi} - \hat{\theta}_S)}}$$

Formula 2.3 – Tajima’s D, where $\hat{\theta}_{\pi} = \hat{\pi}$ and $\hat{\theta}_S = S / \sum_{i=0}^{n-1} (1/i)$, and S is the number of segregating sites in the sample. The limits of confidence intervals around D may be found in Table 2 of Tajima's paper (Tajima 1989a) for different sample sizes. The significance of the D statistic is tested by generating random samples under the hypothesis of selective neutrality and population equilibrium, using a coalescent simulation algorithm adapted from Hudson (1990). The P value of the D statistic is then also provide a parametric approximation of the P-value assuming a beta-distribution limited by minimum and maximum possible D values (see Tajima 1989a). Note that significant D values can be due to factors other than selective effects, like population expansion, bottleneck, or heterogeneity of mutation rates (Tajima 1993; Aris-Brosou and Excoffier 1996; or Tajima 1996).

A series of coalescent simulations with the software Bayesian Serial Simcoal were used to statistically compare descriptor estimates obtained for the invading Mediterranean populations with the original Red Sea populations (Anderson et al 2005; Excoffier et al 2000), given the different sample sizes for each population. This hypothesis testing framework was substantially analogous to a temporal analysis of demographic variation, where the Red Sea populations were assumed as the pre-bottleneck populations and the Mediterranean populations

were assumed to be post-bottleneck populations (Chan et al 2006). A value of 810 generations (81 y times an average values of 10 generations per year) before the present was set for the Red Sea populations, based on the first record of *S. rivulatus* in the Mediterranean Sea in 1929 (Golani 1990). The estimate of generation time was based on an estimated period of 30 days for egg hatching in monogenoidean parasites (Hirazawa et al 2010). The statistical distributions of H, π , and Tajima's D in the simulated population having the same Θ_s (For. 2.4), mutation rate, and transition / transversion ratio as the Red Sea populations, but the sample size of the Mediterranean populations was calculated under a hypothesis of population stability and compared with the observed values.

$$\Theta_s = 4Nu$$

Formula 2.4 – Genetic diversity (Θ_s), where N is the population size and u is the mutation rate, as defined by Watterson, 1975.

We used a prior estimate of the mutation rate of the CoxI gene in *Gyrodactylus* found by Meinilä et al (2004), which was

set with a uniform distribution of [13.7, 20.3]%/ My. We derived the observed effective population size N_e from the observed values of Θ_s (Watterson 1975) using the two values of the mutation rate indicated above.

A model compatible with an HKY model was set without gamma correction, as indicated by the Akaike criterion implemented in ModelTest 3.7 (Posada and Crandall 1998). We set the significance levels, as a minimum of 5% in the simulated data for H and π and a maximum and minimum of 2.5% for Tajima's D , based on a hypothesis of a prolonged or severe bottleneck in recent times. Alternatively a selective sweep may determine low values of both H and π (Grant and Bowen 1998; Avise 2000). Tajima's D tends to become positive with bottlenecks and balancing selection, but negative with directional selection or population expansion (Tajima 1989a and b; Simonsen et al 1995; Nielsen 2001).

3. RESULTS

3.1 Morphological identification

Based on haptoral structures, comprising transverse bars, anchors and hooks, which are characteristic of each species within the *Glyphidohaptor* genus, the 197 helminths specimens, collected from the gill arches of marbled spinefoot, *Siganus rivulatus*, were morphologically identified as *Glyphidohaptor plectocirra* Kritsky et al 2007, belonging to the Dactylogyridae family. Pictures of key structures morphology are reported in the figure below (Figs. 3.1).

Fig. 3.1 – Haptoral structures, comprising transverse bars, anchors and hooks, represent the key-structures for the morphological identification of monogenoidean parasite. The figure shows *Glyphidohaptor plectocirra* sclerites prepared through protease K technique: a) haptoral region; b) entire body; c) haptor – two transverse bars, two couples of anchors and 7 couples of hooks; d) ventral anchor; and e) dorsal anchor (following page).



3.2 Genetic variability and phylogeny

Alignment of a 688 bp portion of the 197 mtDNA CoxI gene sequences indicated the presence of 58 haplotypes and 65 polymorphic sites (27 parsimony informative), and no gaps were observed. This pattern of variability indicates the presence of shallow divergence between haplotypes. Overall, many private haplotypes (Tab. 3.1) were characterised in all populations, particularly those from the Red Sea. The Minimum Spanning Network of haplotypes produced a clear star-like phylogeny (Fig. 3.2), with a central dominant haplotype *10I* that was highly frequent in all populations, and many derived haplotypes that were often present as single copies. Other abundant haplotypes (*12I*, *22I*, *102S*, *153R*) were shared between Red Sea and Mediterranean populations. In contrast, a distinct lineage (haplotype *25IS* and the derived *292E*, *296E*, *332E*, *307E* and *282E* ones) was found exclusively in Red Sea populations, while the basal haplotype *25IS* was shared between Nabq and Eilat populations, and derived haplotypes were unique to the Eilat population.

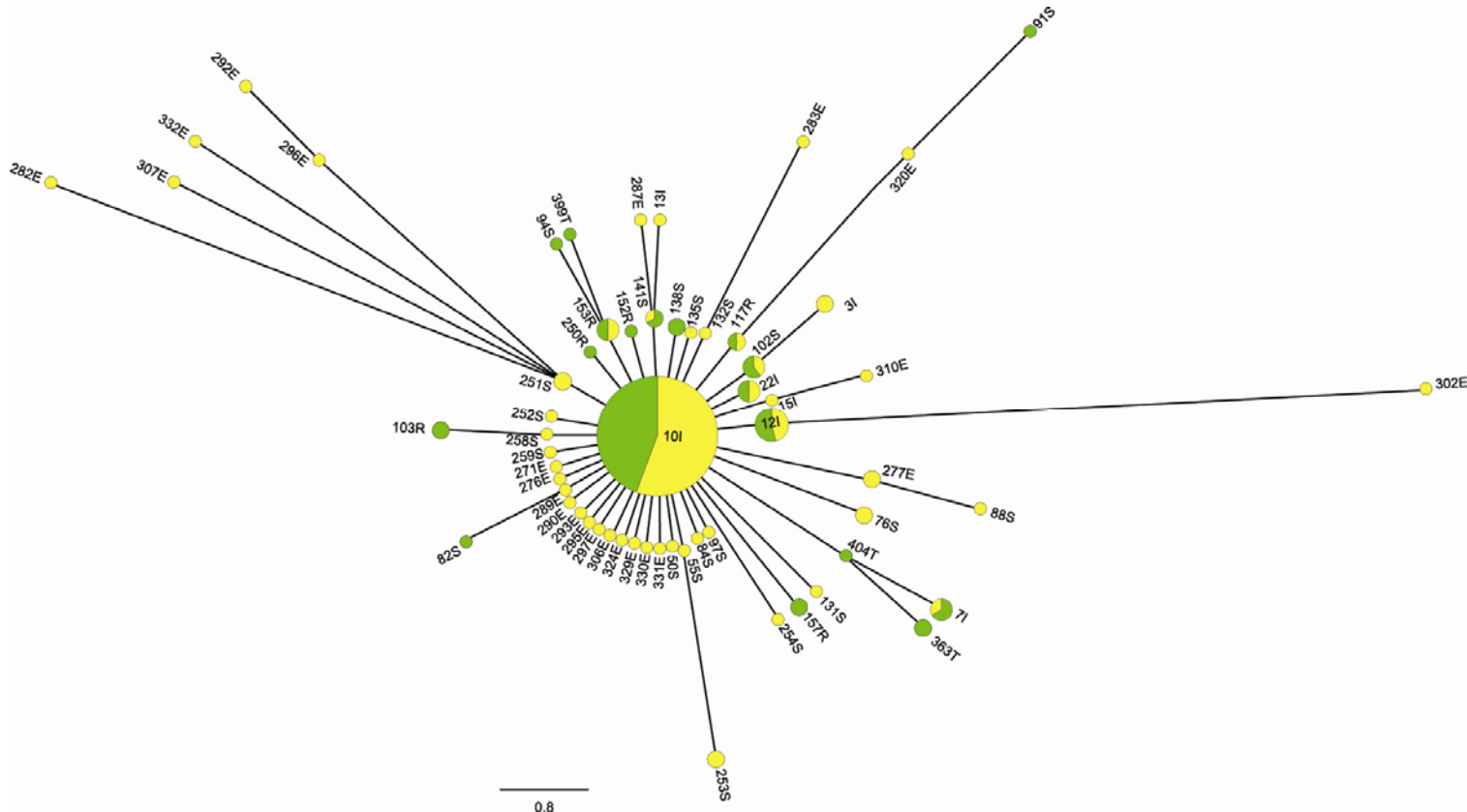
Population		n	n° hapl.	priv. hapl.	H ± s.d.	π ± s.d.	D
Red Sea	Ismailia	17	7	2	0.81 ± 0.079	0.27 ± 0.18	-1.33
	Nabq	52	26	18	0.86 ± 0.045	0.26 ± 0.02	-2.44
	Eilat	53	27	22	0.76 ± 0.035	0.32 ± 0.20	-2.60
Med.	Rhodes	54	12	3	0.60 ± 0.077	0.13 ± 0.01	-2.20
	Tel Aviv	21	6	3	0.61 ± 0.11	0.19 ± 0.14	-1.33

Table 3.1 – Number of samples (n), haplotypes (n° hapl.), private haplotypes (priv. hapl.), haplotype diversity (H), nucleotide diversity (π) and Tajima's D (D) estimated for the five *G. plectocirra* populations. In bold the significant values ($p < 0.05$) of the indices are indicated.

Several trials were conducted on a subset of samples to amplify the ribosomal DNA 18s using the two primers WormA and WormB, but no satisfying results were obtained. However, the terminal region of the 18S gene of the same subset of samples, that include the entire ITS1 and a portion of the 5.8S gene, was then successfully amplified using S1 and IR8 primers. The 681 bp alignment of 60 rDNA sequences, obtained through this second protocol of amplification, showed only two polymorphic sites, which distinguished only three haplotypes (data not shown). Thus, the low variability of this marker at the species level in the monogenoidean parasites was confirmed (Meinilä et al 2004), and any downstream analysis was performed only on the CoxI gene.

All the sequences were deposited in the EMBL database under the codes from HE574491 to HE574550 for the CoxI gene and from HE601931 to HE601933 for the rDNA.

Fig. 3.2 - Minimum Spanning Network among CoxI haplotypes of *Glyphidohaptor plectocirra*. Each haplotype is denoted by a circle indicative of their overall frequency and the relative proportion of occurrence in Red Sea (pale grey) and in Mediterranean Sea (dark grey) is reported.



3.3 Population variability and demographic analysis

Analysis of Molecular Variance tested a hypothesis of genetic structure between the Red Sea and Mediterranean populations, but it also tested the partitioning of variance due to the variability of populations in each sea and between populations regardless of their location (Tab. 3.2).

Uppermost hierarchy level		df	Sum of squares	Covariance component	% of molecular variance	F _{ST} (P)	F _{SC} (P)	F _{CT} (P)
Red Sea/ Mediterranean populations	among groups	1	0.94	0.00741	0.90	0.013 (< 0.01)	0.022 (< 0.01)	0.009 (> 0.01)
	among populations within groups	3	4.30	0.01785	2.17			
	within populations	192	155.78	0.81131	96.93			

Table 3.2 – Hierarchical Analysis of Molecular Variance based on CoxI gene of *Glyphidohaptor plectocirra* from the Red Sea and the recently founded Mediterranean populations.

The results of AMOVA (Tab. 3.2) indicated the absence of any significant structure between the Red Sea and Mediterranean Sea populations, which was congruent with the recent colonization of the Mediterranean Sea and the same overlap of haplotype composition between the two populations of *Glyphidohaptor plectocirra*. Most of the variance was related to variability within populations. A significant partitioning of haplotypes between populations among groups was also detected, although this source of variability explained only about 2% of the total variance.

The estimation of pairwise divergences between populations based on F_{st} (Tab. 3.3) showed contrasting and interesting patterns. Divergences were generally low, but the Eilat population was significantly divergent from all other populations. The Ismailia population, which may be considered as the most complete representative sample of haplotypes likely to colonize the Mediterranean Sea because it is located along the Suez Canal, was not significantly different from the other two Mediterranean populations. However, an unexpected significant divergence was found between this population and the Nabq and Eilat ones located in the Red Sea (but in the Gulf of Aqaba).

Finally, the two Mediterranean populations were not significantly divergent, while the Nabq population showed more affinity with the Mediterranean populations than other Red Sea populations.

		Red Sea			Med.	
		Ismailia	Nabq	Eilat	Rhodes	Tel Aviv
Red Sea	Ismailia	-				
	Nabq	0.022	-			
	Eilat	0.030	0.014	-		
Med.	Rhodes	0.028	0.005	0.016	-	
	Tel Aviv	0.016	0.007	0.022	0.024	-

Table 3.3 – Matrix of pairwise F_{st} estimated between the five *Glyphidohaptor plectocirra* from Red Sea and Mediterranean Sea. In bold the significant values ($p < 0.05$) are indicated.

Hypothesis testing for the occurrence of a bottleneck in populations invading the Mediterranean Sea used only the Ismailia population as a native reference. This was based on an indication that populations from the Gulf of Aqaba might not contribute significantly to invasive gene flow.

The values for haplotype diversity (H), nucleotide diversity (π), and Tajima's D are shown in Table 1. Both the Mediterranean populations showed slightly lower values of H and a more marked decrease in π values, while Tajima's D was negative in all cases with significant values ($p < 0.05$) for the Nabq, Eilat, and Rhodes populations.

A series of serial coalescent simulation were used to test whether the observed differences were due to an effective reduction of N_e during colonization. The Ismailia population was considered as an appropriate original population that colonized the Mediterranean Sea. Its evolution was simulated under the hypothesis of demographic stability through 810 generations, based on a sample size equivalent to the Mediterranean populations analysed. Basing on the Ismailia Θ_s estimate and the chosen mutation rates, values of N_e comprising between ~ 50000 and ~ 100000 individuals were used for these simulations, assuming that this parameter determined mostly the overall genetic variability of the simulated genealogies. The *a posteriori* distributions of H, π , and Tajima's D were compared with the observed values in the Mediterranean populations. The estimated

a posteriori distributions for the three parameters are shown in figures below (Fig. 3.3, 3.4 and 3.5), with sample sizes equivalent to those of the two populations of Rhodes and Tel Aviv. As expected, the distributions of H and π showed a shift towards higher modal values with increasing population size, whereas Tajima's D was almost insensitive to this change. The raggedness of the parameter distributions was mainly influenced by the sample dimension, especially for H , which was more pronounced in the histograms obtained for $n = 21$ (sample dimension of the Tel Aviv population).

The difference of the observed values of H and π from simulation distributions was not significant for both Mediterranean populations, which indicates the absence of any change of genetic variability in invading populations related to pronounced gametic sampling or N_e lowering. In contrast, Tajima's D was significantly lower for both the simulated population dimensions in the case of the Rhodes population, and almost significant for $n = 50000$ in the case of the Tel Aviv population. Thus, a pattern of directional selection may be acting on the invading populations.

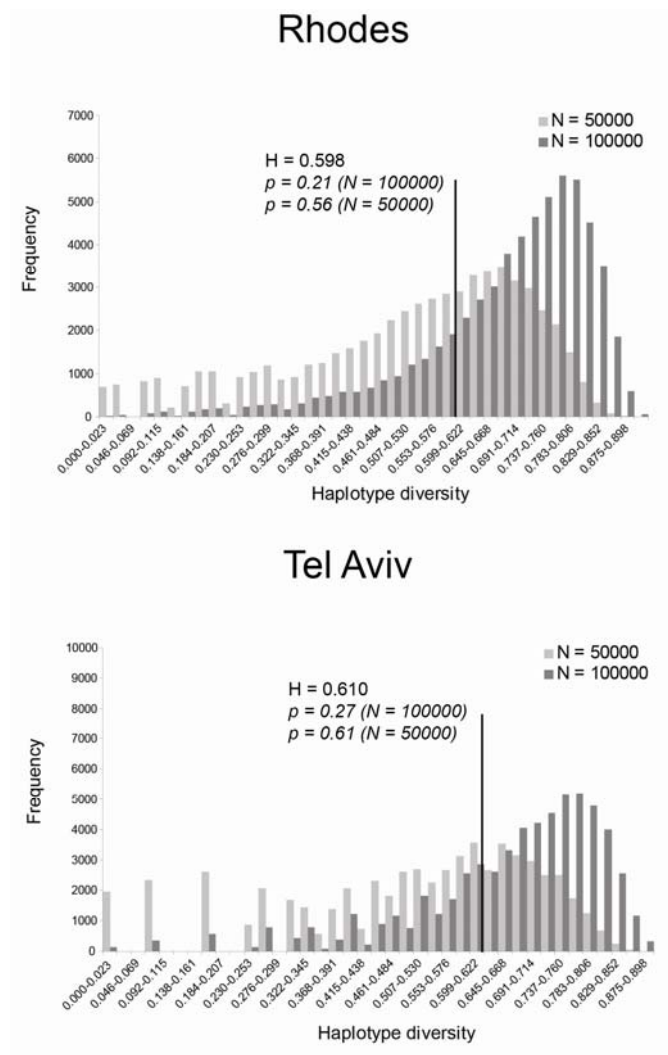


Fig. 3.3 - Distribution of simulated values of haplotype diversity under a coalescent approach setting a constant population size and the genetic variability parameters derived from the Ismailia population, but the sample size of Rhodes or Tel Aviv populations. For each simulation, two run were performed by imposing two different effective population sizes ($N_e = 50000$ and $N_e = 100000$). The measured values for both Rhodes and Tel Aviv populations are indicated in the histograms, together with their p-values respect to the simulated distributions.

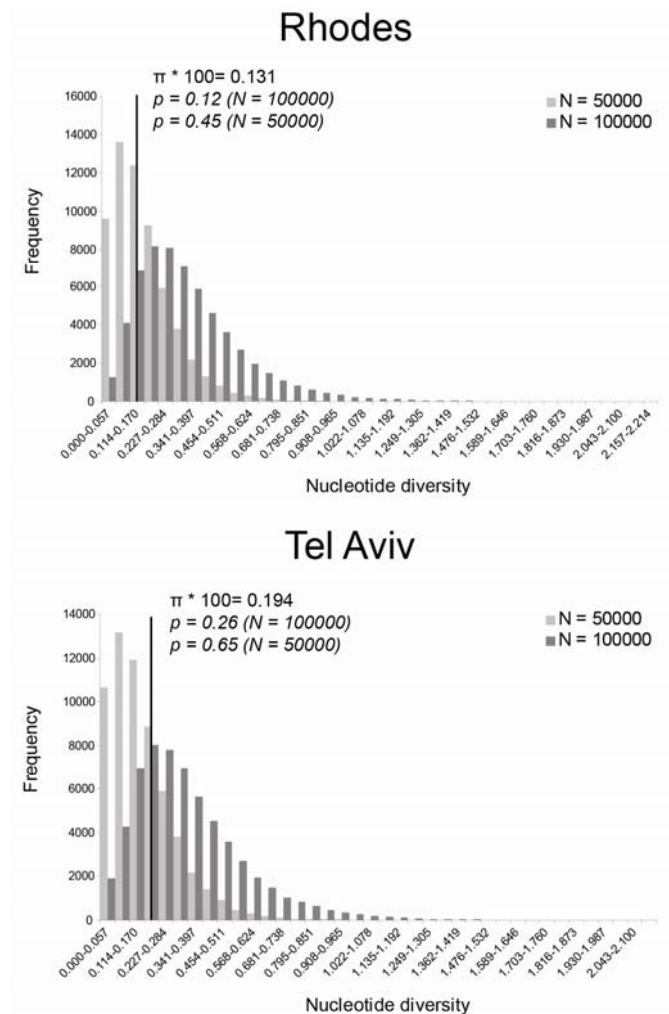


Fig. 3.4 - Distribution of simulated values of nucleotide diversity under a coalescent approach setting a constant population size and the genetic variability parameters derived from the Ismailia population, but the sample size of Rhodes or Tel Aviv populations. For each simulation, two run were performed by imposing two different effective population sizes ($N_e = 50000$ and $N_e = 100000$). The measured values for both Rhodes and Tel Aviv populations are indicated in the histograms, together with their p-values respect to the simulated distributions.

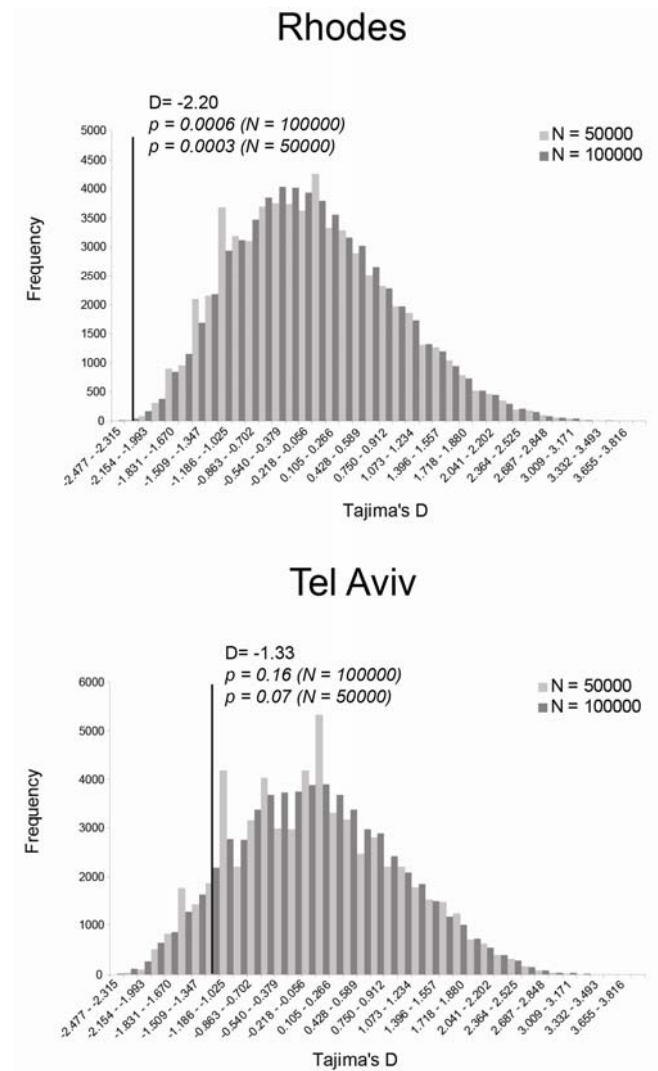


Fig. 3.5 - Distribution of simulated values of Tajima's D under a coalescent approach setting a constant population size and the genetic variability parameters derived from the Ismailia population, but the sample size of Rhodes or Tel Aviv populations. For each simulation, two run were performed by imposing two different effective population sizes ($N_e = 50000$ and $N_e = 100000$). The measured values for both Rhodes and Tel Aviv populations are indicated in the histograms, together with their p-values respect to the simulated distributions.

4. DISCUSSION

Coalescent simulation analysis indicated no bottleneck or founder effects in *Glyphidohaptor plectocirra*. Nevertheless, a signal of directional selection was more apparent with distance from the colonizer source, which was detected along with a significant genetic structure between the Red Sea populations. The *G. plectocirra* pattern shares partial similarities with that found in their hosts (Azzurro et al 2006; Hassan et al 2003; Bonhomme et al 2003).

4.1 Directional selection

The absence of genetic structure between invading and donor populations of *Glyphidohaptor plectocirra* is congruent with the pattern observed in their hosts. In fact, high propagule pressure during the initial colonization and a constant or repeated gene flow from the Red Sea to the Mediterranean area is thought to have characterized the invasion of siganids in the Mediterranean Sea (Azzurro et al 2006; Bonhomme et al 2003; Hassan et al 2003; Golani 1990) (Tab. 4.1).

		<i>H</i>	π	<i>D</i>	Main inference
<i>Siganus rivulatus</i>	Red Sea	1.00	0.91	-1.42	
	Mediterranean Sea	0.85	0.53	Not significant	No bottleneck
<i>Glyphidohaptor plectocirra</i>	Red Sea	0.81	0.28	-2.12	No bottleneck
	Mediterranean Sea	0.61	0.16	-1.77	but directional selection

Table 4.1 – Overall comparison between haplotype diversity (*H*), nucleotide diversity (π) and Tajima’s *D* (*D*) of *Siganus rivulatus* and *Glyphidohaptor plectocirra* populations from Red Sea and Mediterranean Sea.

Our results contribute to understanding the factors that influence the genetic structure of invasive parasites, especially host dispersal (Miura et al 2006).

Given the rejection of the bottleneck hypothesis, the apparent diminution of haplotype and nucleotide diversity estimated for Mediterranean populations should not be considered as related to demographic constraints. Other factors, such as directional selection, could have shaped the genetic variability of *G. plectocirra*. A cline of selection, acting as an adaptive evolutionary driver in the face of gene flow (Vellend et al 2007), was evident from Tel Aviv to Rhodes populations, which correlated with their progressive genetic divergence from

the source population (Ismailia), based on the F_{st} evidence. In evolutionary terms, a progressive genetic differentiation between the Red Sea and Mediterranean populations may be likely, although the level of intrapopulation genetic diversity will be substantially maintained. What mechanisms might explain the discrepancies in evolutionary diversification between invasive hosts and their associated parasites? It is recognized that environments can alter the strength of selection on both host and parasite genotypes, but interactions may occur between any combination of host genotype, parasite genotype, and the environment (Wolinska and King 2009). Parasite genotype \times environment interactions may have influenced *G. plectocirra* fitness independently of the host genotype. Strong environmental gradients exist in the Mediterranean Sea, which are basically determined by the increasing temperature when moving roughly from south-east to north-west (Bianchi 2007), and this may have exerted a primary selective role on *G. plectocirra* genotypes. However, various components of host-parasite fitness are commonly affected by the environment (Wolinska and King 2009), and tolerance to temperature may not be the only factor responsible for selection.

Host genotype x environment (*i.e.*, increasing the resistance of hosts to some genotypes) and host genotype x parasite genotype interactions (*i.e.*, the fitness of parasites is maximised in specific matches of host and parasites genotypes) appear less likely, since no selection was detected in the hosts and any bottleneck was not detected in both hosts and parasites (Azzurro et al 2006; Bonhomme et al 2003; Hassan et al 2003).

The extension of this analysis to other more distant populations in the Mediterranean basin, coupled with an analysis of life trait-linked loci, may further emphasize these evolutionary factors, which may be indicative of stronger genetic differentiation between the source and Mediterranean populations, rather than within the same heterogeneous Mediterranean basin.

4.2 Anisotropic source of invading haplotypes

Characterization and comparison of genetic variability of native and invading populations relies heavily on key parameters when trying to take into account the true pattern of dispersal

followed by an exotic species, such as which source populations should be included in the data set. It has been demonstrated (Dlugosch and Parker 2008; Hierro et al 2005) that the knowledge of the original genetic structure and biogeographic history of native populations constitutes an unavoidable step in the framework of a comparative study of invading species. In this study, the peculiar biogeographical setting was a significant aid to the application of the theoretical model on which the bottleneck hypothesis test was based. Lessepsian dispersals are necessarily constrained by the pathway of the Suez Canal, where the origin of migrating specimens is located. The decision to collect one population sample along the Suez Canal (*i.e.*, the Ismailia population) allowed comparison of the genetic variability of single Red Sea populations with the most realistic representative samples of the invading haplotypes, which provided a description of the genetic structure of invading haplotypes. Analysis of genetic divergence between populations, based on F_{st} index and AMOVA, supported this theoretical model, showing a lack of divergence of the Ismailia populations from the two Mediterranean ones and a parallel divergence from other Red Sea populations.

The shallow phylogenetic structure of haplotypes indicates a recent history of divergence and demographic expansion within the original Red Sea population, probably linked to the end (about 10000 ybp) of strong environmental variations that occurred during the last glacial phases when the Straits of Bab Al Mandab were closed and salinity increased throughout the Red Sea (Shaked et al 2002). The negative values of Tajima's D in all Red Sea populations and those from the Gulf of Aqaba were significant, while the high values of H compared to estimates of π support the hypothesis of recent demographic expansion. This scenario constitutes the background against which the genetic variability of the Mediterranean populations was compared.

Despite the recent history of divergence in the Red Sea, patterns of population isolation and drift were detected between the Ismailia and Gulf of Aqaba populations. Despite the low number of populations included in our analysis, the data were sufficient to highlight the marginal role that populations from the Gulf of Aqaba play in the invasion process, particularly the Eilat population which was significantly divergent from all other populations and hosts a unique lineage. The oceanographic

conditions acting on the Red Sea favour gene flow in towards the Gulf of Aqaba but not outwards (Kochzius and Blohm 2005; Berman et al 2000) during the larval stages, and the persistence of newly generated haplotypes was caused by the presence of gyres along its main axis. In the case of *Atherinomorus lacunosus*, Bucciarelli et al (2002) hypothesized the exclusion of Northern populations in the Gulf of Aqaba from the pool of individuals that colonized Mediterranean regions. These findings emphasize the need to extend sampling activities outside the Gulf of Aqaba, which is a traditional location for many studies, when dealing with population genetics study of Lessepsian species.

The presence of unique haplotypes in the Mediterranean populations suggests that our Red Sea samples did not encompass all sources, or that they could not have been sampled by chance in the Red Sea populations with such a low sample size, particularly in Ismailia. Considering the low number of unique haplotypes and the fact that migration flow had to pass through the Suez Canal, the second hypothesis appears more likely. An alternative explanation might be that some haplotypes were rare in the native area and their frequency increased during

introduction and subsequent invasion. This scenario seems less parsimonious than the existence of unsampled haplotypes, but the presence of directional selection may argue in favour of this. A third possible explanation involves the *in situ* emergence of novel haplotypes following introduction, but this hypothesis appears even more unlikely. The time scale of Lessepsian invasions is restricted to the last ~100 years, and the evolution of new haplotypes appears highly improbable given the mutation rates at the locus studied (13.7–20.3% / My).

5. CONCLUSIONS

The genetic flow relationships between five populations was verified, three from the Red Sea (Nabq and Ismalia, Egypt, and Eilat, Israel) and two from Mediterranean Sea (Rhodes Island, Greece and Tel Aviv, Israel). The results of the present study demonstrate the absence of a genetic bottleneck in Mediterranean populations of *Glyphidohaptor plectocirra*, which is congruent with that of their hosts, *Siganus rivulatus* and *Siganus luridus*. This strict association between invading hosts and their parasites indicates a pivotal role for host dispersal on the size of parasite propagule pressure. Nevertheless, novel information has emerged, such as the presence of a directional selection signal differentiating the response of the parasite to the newly invaded environment with respect to their hosts. The scale and direction of this selection cannot be argued until a detailed and specific analysis has been performed with non-neutral markers. Our study also gave some indication of an adaptive cline. In the future, the analysis of other Mediterranean populations could help to clarify the role played by environmental or ecological gradients in shaping the genetic variability of exotic parasites.

Finally, this study also provides indications of an anisotropic contribution of Red Sea lineages to the colonizing gene flow, which highlights the need to identify the true source populations as accurately as possible when studying invasion dynamics.

Our purpose for the future is, however, in addition to the identification of a non-neutral marker in order to identify intensity and direction of observed selection, the development of highly variable markers with greater resolving power, such as microsatellites (actually not available for monogenoidean parasites), that could provide more insight on the biogeographic patterns, the genetic structuring and the reproductive strategies of this species over a range of spatial and temporal scales.

ACNOWLEDEGMENTS

First of all, I want to thank Fabrizio Stefani (University of Milan Bicocca, during my PhD, and, now, Consiglio Nazionale delle Ricerche - CNR) for his fundamental support in statistical analyses and his contribution in the production of this work and associated paper.

I'm really grateful to Paolo Galli, my tutor, who allowed me to complete my PhD, even if I unexpectedly escaped from the University some time ago. The reasons of my choice are so many and so complicated that I could need a second PhD thesis in psychology to explain them extensively and surely none is interested about.

I'm also grateful to Ernesto Azzurro (ISPRA) for the support about rabbit fish biogeography and ecology and to Angelo Colorni (Department of Pathobiology, National Center for Mariculture, Israel Oceanographic and Limnological Research) for collection of Israel samples.

Also, I cannot forget to thank students Stefano Bonelli, Laura Bernabò, Davide Parise and Andrea Guastamacchia for their help in lab, and the Egyptian Environmental Affairs Agency rangers in Nabq, Egypt, for their help in sampling my Egyptian fishes.

I also want to thanks Simone, Davide, Roberto and Francesca for all the fantastic unforgivable days spent in and outside the lab.

At the end I have surely to mention my wife, Donatella, my parents, Pino e Marilede, my brothers, Paola and Gabriele, and all the people who helped me to find the time to write this work, also during these last months, in which I was often so busy to forget to spend some words or some time with them all.

In think that's all and, if someone who helped me has not been mentioned, it's only because I have just written these few words in ten minutes before the submission of this thesis.

Grazie, *Giovanni*

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