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Microsatellite variation and reproductive interactions of common and Egyptian soles in Mediterranean sympatric demes

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Microsatellite variation and reproductive interactions of Common and Egyptian soles in Mediterranean sympatric demes

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In the Mediterranean, the common (*Solea solea*) and Egyptian (*S. aegyptiaca*) soles are two of the most valuable flatfish fishery resources. In the past, *S. aegyptiaca* was erroneously synonymised with *Solea solea* because of the great similarity of the external rough morphology (i.e. cryptic species). Recently, the fish biologists have proven species distinctiveness mainly using mitochondrial DNA markers; however mtDNA-based molecular test can not suitable for detecting reproductive interactions among them. The common and Egyptian soles seem to co-occur in several areas of the Mediterranean forming sympatric demes. This wide sympatric distribution and the close phylogenetic relationship between the two sole species will allow a scenario for potential ecological and evolutionary interactions.

The identification and assessment of ecological and reproductive interactions of cryptic species have important implications for sustainable management and conservation of fishery resources, because different species can differently respond to environmental pressures and changes (Bickford et al., 2006); as well, these goals are important for species already considered endangered or threatened because they might be composed of multiple species that are even more rare than previously supposed (Schönrogge et al., 2002). In addition, the human activities (e.g. aquaculture and breeding programmes) have increased the risks of ecological relationships between wild and domesticated populations, as they might play significant role in the natural process of adaptation, local extinction/recolonization events, hybridization or disrupting natural selection effects. The highlighting of such ecological and genetic interactions between these two sole species in the Mediterranean basin enables the understanding of processes such as population divergence, speciation and hybridization that can create evolutionary novelty.

This thesis is a part of the EU FP7 Project "*The Structure of Fish Populations and Traceability of Fish and Fish Products"* (FishPopTrace). The thesis aims to advance in the taxonomic, zoogeographic, ecological and evolutionary knowledge on the Mediterranean soles i) by developing a multiplex PCR test for the rapid screening of the two cryptic species, ii) by analysing the species composition of several geographical

demes and iii) the possible occurrence of interspecific hybridization and/or allele introgression in mixed populations, using single-locus and multi-locus genetic assignment tests based on nuclear codominant markers as internal transcribed spacer of ribosomal DNA genes and microsatellite loci, respectively.

Sole individuals (N = 179) were collected in 2009, using commercial vessels, from four sampling sites in the Mediterranean: Viareggio, Lagoons of Cagliari (South Sardinia), Akdeniz and Antalya (Turkish coasts), and Alexandria (Egypt). All the specimens were genotyped at eight neutral microsatellite loci and at the Internal Transcribed Spacer 1 locus of the ribosomal RNA genes. Among them, 125 individuals were previously assigned to putative sole species by cytochrome b mtDNA haplotype.

The analysis of species composition in the four population samples revealed that Lagoons of Cagliari and Turkish coasts are mixed demes where the two sole species were sympatric. On the other hand, Viareggio sample was composed uniquely by *S. solea* and Alexandria was almost completely formed by *S. aegyptiaca* (only one *S. solea* individual, likely a migrant from the Turkish coasts, was found in this sample). The wider distribution of *S. aegyptiaca* in the Mediterranean and its frequent sympatry with *S. solea* lead to argue that the two species have been frequently misidentified. Further multidisciplinary data (i.e. combining data from morphology, reproductive biology and life history with genetic data of the same individual) obtained from new and more samples are needed to unravel distribution and ecology of *S. aegyptiaca* in the Mediterranean and its ecological interactions with the cryptic species *S. solea*.

All the analytical approaches and microsatellite datasets consistently revealed a clear genetic separation of the two species. The multilocus F_{st} estimate and almost all single-locus F_{st} values were high and significant indicating a complete lacking of gene flow among taxa. Individual multilocus genotypes were grouped by PCA and clustered by the Bayesian clustering method in two well-distinct groups corresponding to the putative species. These results ruled out definitely the occurrence of any past and present hybridization events between these two sole species at least in the geographical demes I have analysed. These findings were consistent with previous outcomes from Borsa et al. (2001) and She et al. (1987a) that have argued reproductive isolation between Mediterranean *S. solea* and *S. aegyptiaca.*

The panel of 8 microsatellite loci which can cross-amplify in the two *Solea* species can be considered one of the most versatile and powerful set of molecular markers for resolving ecological and evolutionary questions at multiple taxonomic levels (from

species to populations and individuals) in this group of flatfish species. From one to few microsatellite loci were suitable and powerful for the accurate and reliable identification of sole species. Even though the multiplex PCR ITS1 assay didn't perform completely in the identification of hybrids between *S. solea* and *S. aegyptiaca* or viceversa, it has been proven as a rapid, costless and valuable tool for the *Solea* species identification, being its results 100% consistent with the microsatellite-based species assignment.

All in all, using available and newly developed molecular markers (i.e. a panel of microsatellite loci and the ITS1 locus, respectively), this thesis work has improved species identification by developing rapid and discriminating PCR-based tests and the understanding of ecological and evolutionary relationships between these two species. The technological and scientific advances can be used for improving the sustainable exploitation of these two fishery resources in the Mediterranean.

1. INTRODUCTION

1.1. The Fish Pop Trace project

This thesis work is a part of the Small or Medium-Sized Research Project "*The Structure of Fish Populations and Traceability of Fish and Fish Products*" (FishPopTrace), an EU FP7 aiming at the development of traceability tools for an aware management of four commercially important fish species of the European seas: herring (*Clupea harengus*) and cod (*Gadus morhua*) in the FAO fishery area 27 - NE Atlantic, and hake (*Merluccius merluccius*) and common sole (*Solea solea*) in both FAO fishery areas 27 and 37 – Mediterranean and Black Sea (Stockstad, 2010).

Today, the economical activities such as fishery and aquaculture can't leave aside a responsible management and legal exploitation of the fish stocks. The goal of FishPopTrace is to increase the knowledge of the status of bio-economically important fish species using an explorative research approach.

Human-dominated marine ecosystems are experiencing an accelerated loss of diversity at both population and species levels, with largely unknown consequences (Worm et al., 2006). The FAO estimates that today 80% of marine fish stocks are fully or overexploited worldwide, a dire situation which is further aggravated by the continuously increasing demand of fish and fish products. Additionally the fishing sector is penetrated by an extremely high level of illegal fishing activities (Stockstad 2010). Illegal, unreported and unregulated (IUU) fishing not only threaten marine ecosystems and habitats, obstruct sustainable fisheries and has highly negative socio-economic consequences, but also deeply impedes scientific fisheries assessment. Thus IUU fishing contributes to the overexploitation of fish stocks and is a hindrance to the recovery of fish populations and ecosystems (Stockhausen and Martinsohn, 2009).

In fisheries management the concept of "stock" is a key-concept: stock is arbitrary group of fishes large enough to be essentially self-reproducing, with all members, of the same species, having similar life history characteristics and living in the same area (Hilborn and Walters, 1992; King, 1995). Thus, understanding stock structure of harvested species and how fishing effort and mortality are affecting stock features it's crucial (Grimes et al., 1987) and critical to design appropriate fishery management strategies when multiple stocks are differentially exploited (Ricker, 1981). In fact, fishing is the dominant factor reducing populations and fragmenting habitats of marine species and is predicted to leading to local extinction events, especially among large,

long-lived, slow-growing species and endemic species (Millenium Ecosystem Assesment 2005). Habitat fragmentation (i.e. the reduction of natural cover into smaller and more disconnected patches) compounds the effect of habitat loss (Millenium Ecosystem Assessment 2005). For this reason it is of priority importance to known the present status of the fish stocks, in order to construct management plans that take into account all biological, ecological and taxonomical data of the target species.

1.2. Target species

1.2.1. Classification and morphology

The common sole *Solea solea* (Linnaeus, 1758) or *Solea vulgaris* (Quensel, 1806), and the Egyptian sole *Solea aegyptiaca* (Chabanaud, 1927) are flatfishes (genus: *Solea*, family: Soleidae, order: Pleuronectiformes, class: Actinopterygii). As all flatfishes they're asymmetrical, with a flat-shape body, lying on the bottom on the left side of the body, and, as all species belonging to Soleidae family, with both eyes on the right side. The eyed side has a mimetic pigmented livery from greyish brown to reddish brown, whereas the blind side is white, without any pigmentation.

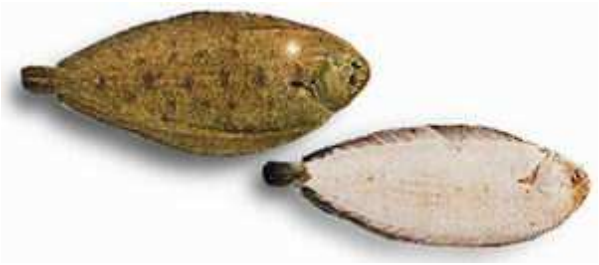


Fig.1.1: Appearance of the Common sole (*Solea solea*) individual at the ocular right (above) and blind left (below) sides (http://www.fao.org/fishery/species/3367/en)

Solea solea and *S. aegyptiaca* are cryptic species; the sympatry of both species in some Mediterranean areas was recently discovered. *Solea aegyptiaca* was considered by Quignard et al. (1984) to be a distinct species from *S. solea* based on allozyme polymorphisms. However, several studies based on genetic and morphometric data (i.e. number of anal fin rays, dorsal fin rays and vertebrae) have debated about this distinction (Quignard et al. 1986; Fischer et al. 1987; Goucha et al. 1987; Tinti and Piccinetti 2000; Mehanna 2007). Borsa and Quignard. (2001), using mtDNA variation, have demonstrated that *S. aegyptiaca* and *S.solea* are distinct species, and reproductively isolated from each other wherever they were found in simpatry. The Egyptian sole is morphologically identical to the common sole, and the only character that enables a distinction between these two species seems to be the number of

vertebrae, 39-44 in *S. aegyptiaca* and 46-52 in *S. solea* (Fischer *et al.* 1987; Tinti and Piccinetti 2000).

Solea solea is genetically differentiated from both the closely related species *S. aegyptiaca* and *S. senegalensis* (Borsa and Quignard, 2001; Vachon et al., 2008), that conversely seem to reproductively interact. Using a multivariate analysis of the electrophoretic polymorphism of Mediterranean *S. solea*, *S. aegyptiaca* and Atlantic-Mediterranean *S. senegalensis* populations, She et al. (1987a) documented the occurrence of hybrid individuals between the Egyptian and Senegalese soles, and confirmed that *Solea solea* is the most differentiated and reproductively isolated ancestral taxon. The reproductive interactions between *S. aegyptiaca* and *S. senegalensis* and the occurrence of hybrids are confirmed also by a more recent work of Ouanes et al. (2011) that resumed and expanded the work done by She et al. (1987a).

1.2.2. Species distribution, biology and ecology

The two soles species seem to have different distributions (Fig.1.2); *S solea* is recorded in the continental shelves of the Eastern Atlantic and Mediterranean, with preferences on sandy and muddy bottoms, from the shore down to 300 m (Rijnsdorp and Witthames 2005). *S. aegyptiaca* seems to be mainly present in the southern and eastern part of the Mediterranean, from the Tunisian to the Turkish and Egyptian coasts (Fisher et al. 1981;

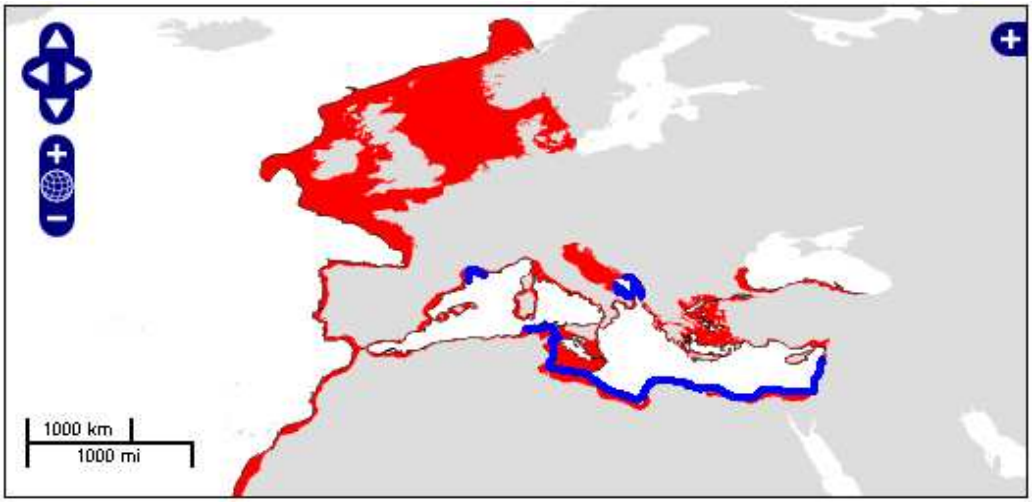


Fig.1.2: Distributions of *Solea solea* (red) and *S. aegyptiaca* (blue);
(from http://www.fao.org/fishery/species/3367/en).

Mehanna 2007), the southern Adriatic coasts and the Gulf of Lions (Borsa and Quignard 2001). Hence, it can be supposed that only in these latter areas, the common and Egyptian soles formed sympatric demes.

Both species are benthic and sedentary. Soles are carnivorous predators that ground on olfactory senses and have nocturnal habits. The sole diet mostly includes invertebrates (polychaet worms, molluscs, small crustaceans) and small fishes (Tortonese, 1975; Fischer et al., 1987).

S. solea can live up to 26 years, and the maturity is reached after the third year (www.fishbase.org); the spawning season occurs generally in winter, showing a peak from December to March in the North Adriatic Sea (Vallisneri et al., 2001), but great

Sole ***Solea solea***

Spawning ground	Spawning season	Maturity Weight*	Maturity Length*
North Sea: Netherlands	April-June	355 g	33 cm
North Sea: Skagerrak & Kattegat	April-June	154 g	25 cm
Irish Coast	May-June	323 g	32 cm
Bay of Biscay	Dec.-May	423 g	35 cm
Mediterranean	Jan-April	173 g	26 cm

Fig.1.3: Variation of reproductive features in *Solea solea* (from http://www.ifm-geomar.de).

variations depending on latitude, photoperiod and temperature (Fig.1.3) are documented by Vinagre et al. (2008) and Vallisneri et al. (2001).

The ecology and life history traits of *S. aegyptiaca* are less documented and this could be related to the difficult to distinct it from the cryptic species *S. solea*. The only available work dealing with age determination of *S. aegyptiaca* was conducted by Ali (1995) along the Alexandria coast, who found the maximum lifespan as 3 years. Length at first sexual maturity was estimated by Mehanna (2007) as 14.2 cm for males and 15.1 cm for females. The spawning season extends from November to May with a peak in January and February (Mehanna, 2007).

From this review, it is apparent that the lack of a clearcut and affordable identification of sole individuals at the species level undermines the reaching of solid and suitable knowledge of the biological and ecological features of these two sole species economically important in the Mediterranean. In addition, because they might be sympatrically distributed in several areas of the Mediterranean, taxonomic uncertainties might lead to record strongly biased fishery data which can prevent correct stock assessments and appropriate management strategies.

1.2.3. Cryptic species

Two or more species can be defined "cryptic" when they share rough external morphology, but are genetically clearly distinguishable (Bickford et al., 2006).

Some authors (Palumbi and Lessios, 2005) suggested that a cryptic species can be morphologically identical to the parental one because of the recent evolutionary divergence. Marine environmental factors might impose stabilizing selection of phenotype acting on morphology, reducing or eliminating morphological changes that can accompany speciation, thus bringing some species to morphological stasis and enabling the "origin" of cryptic or sibling species (Bickford et al., 2006). A sibling species is a cryptic sister species, that is morphologically identical to the closest relative species and hasn't been distinguished taxonomically from that one (Bickford et al., 2006). The cryptic speciation should occur more commonly in those species that based primary biological activities, such as predation and reproductive interactions, on non-visual signals, because changes in these biological features do not necessarily need of parallel morphological changes (Bickford et al. 2006).

As *S. solea* and *S. aegyptiaca* are distinct and philogenetically distant species, and as their primary activity are based on non-visual signals, the Bickford theory (2006) could be the best explanation for these two species, that could hypothetically became cryptic because of evolutive convergence.

Identification of cryptic species and cryptic species complexes has important implications for conservation of natural ecosystems and resources management, because different species can differently respond to environmental pressures and changes (Bickford et al., 2006). In addition, species already considered endangered or threatened might be composed of multiple species that are even more rare than previously supposed (Schönrogge et al., 2002).

1.3. Molecular markers

Molecular markers can give information about dispersal, gene flow, biogeography, kinships and phylogenetic relationships of living organisms (Avise 2004). A basic advantage of molecular markers is that, dealing with cryptic species and unknown species structures, they can make the distinction between analogous (i.e. characters that independently evolved and converged) and homologous traits (i.e. characters that are identical by descent).

Since ecology and evolution necessarily have time components the power of resolution of molecular markers used should match the time scale of interest (Feràl et al, 2002) and the goal of the study.

Molecular markers located in the mitochondrial DNA (mtDNA) are haploid and only maternally inherited, and because of the lack of recombination each haplotype has only one ancestor in the previous generation. Although mtDNA are universal, easy to be isolated and optimized, and highly informative for the reconstruction of evolutionary relationships at multiple taxonomic levels, they are not suitable for solving some evolutionary patterns and processes. For example, the detection of hybridization and gene introgression events is necessarily linked to the use of nuclear DNA codominant markers, which are biparentally inherited and can identify recent or past reproductive admixture among species, races and populations (Buonaccorsi et al., 2001).

In this study, I've used two types of nuclear DNA markers: a panel of microsatellite loci and a single locus corresponding to the Internal Transcribed Spacer 1 of ribosomal DNA genes (ITS1).

Microsatellite (or Short Tandem Repeats, STRs) loci might be good candidates for identification purposes, due to their high variability, codominant diploid inheritance and high discrimination power at the within-species level and at small geographical scales (Rico et al. 1996, Manel et al. 2005). In marine fishes microsatellite loci have high mutation rates, display high levels of variation and provide high statistical power in parentage testing and kinship reconstruction (Wilson and Ferguson, 2002), population identification (De Woody and Avise, 2000) and population assignment (Hauser et al., 2006). Microsatellite variation analysis usually requires the development of species-specific markers, but sometimes loci from closely related species can be used with cross-species amplification success (Maes et al 2006; Hauser and Seeb, 2008).

Ribosomal DNA (rDNA) has both rapidly and slowly evolving regions, and it is particularly useful for phylogenetic analysis (Mindell and Honeycutt, 1990); the slowly evolving coding regions are suitable for comparing distantly related species, while the more rapidly evolving non coding external and internal transcribed spacers (ETS and ITS, respectively) are suitable for resolving evolutionary relationships at low taxonomic levels (Fernandez et al., 2001). Furthermore the multicopy nature of rDNA makes this marker highly sensitive to hybridization because of the accumulation of evidence of past hybridization events (Wyatt et al., 2006).

1.3.1. Microsatellites

Microsatellites are nuclear DNA regions formed by short (1 to 6 bp) tandemly repeated sequences (Fig. 1.4) widespread in both eukaryotic and prokaryotic genomes. They are highly abundant in the intronic regions of eukaryotic genomes. Microsatellite loci are co-dominant and are considered evolutionarily neutral DNA markers. Because of their high level of polymorphism, relatively small size and rapid amplification protocols, they're widely used for population genetic purposes (Bhargava and Fuentes, 2010). Microsatellites can be useful in providing estimates of neutral genetic variation among populations, i.e. variation with no direct effect on fitness and selection not acting upon alleles (Holderegger et al., 2006). As they're not under selection, these markers have mutation rates between 10^{-3} and 10^{-5} mutation/locus/generation; these mutation rates are very high as compared with the rates of punctiform mutations at coding gene loci (Bhargava and Fuentes, 2010).

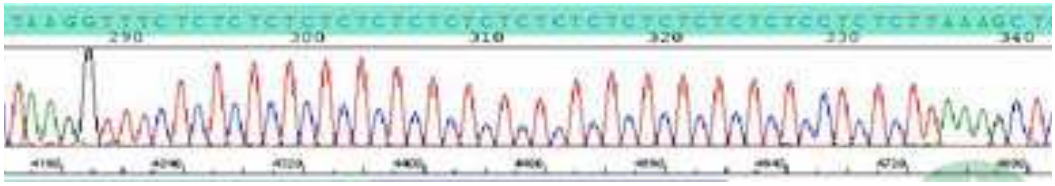


Fig 1.4. Core region of a microsatellite locus represented by several repeats of a dinucleotide $(CT)_n$

Selection can however act on nearby flanking regions, where the primers are designed, affecting, in some cases, the cross-species amplification. Indeed cross-species amplification between more or less distant species is a consequence of highly conserved microsatellite flanking regions (Rico et al., 1996; O'Connell and Wright, 1997).

1.3.2. Internal Transcribed Spacer 1

The internal transcribed spacer 1 (ITS1) is a non-coding region located between the conservative 18S and 5.8S rRNA genes. The ribosomal DNA genes are arranged in multiple tandem repeated units, separated one each other by non transcribed spacers (NTS) (Fig 1.6).

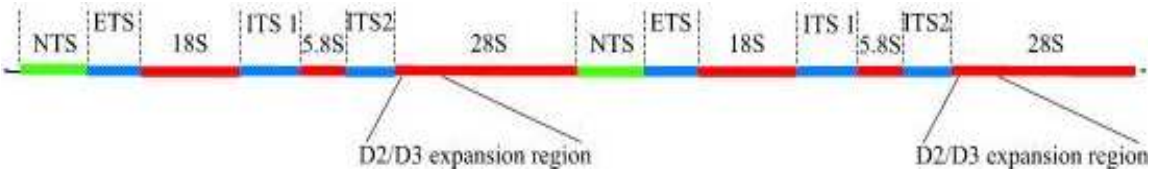


Fig.1.6.: The organization of ribosomal DNA genes

Designing primers on the nearby flanking 18S and 5.8S coding regions it's possible to cross-amplify the ITS1 fragment in also highly distant species, because 18 S and 5.8S are very conserved regions as they're under selection: this feature makes the ITS1 an universal powerful marker. The ITS1 region evolves rapidly but the homogenizing forces of concerted evolution and molecular drive (Arnheim, 1983; Dover, 1986) are believed to minimize the degree of intraspecific variation, and make the ITS region suitable for phylogenetic comparisons among closely related taxa. The ITS1 has been used for phylogenetic studies in a very wide variety of organisms, and also in fish systematics (Pleyte et al., 1992; Phillips et al., 1994; Domanico et al., 1997; Sajdak and Phillips, 1997; Booton et al., 1999; Huyse et al., 2004; Chow et al. 2006). Though successful in resolving conflicting trees derived from nuclear and mitochondrial DNA data in salmonids (Pleyte et al., 1992; Phillips et al., 1994; Domanico et al., 1997; Sajdak and Phillips, 1997) or providing new insights for complicated cichlid evolution (Booton et al., 1999), little attention has been paid to the intraspecific variation of the ITS region in these studies.

2. AIM

The environmental and maritime policies of European Union stretch to a responsible management of the fishery resources to improve sustainable exploitation of commercial stocks and the conservation of biodiversity in harvested ecosystems. The EU FP7 project FishPopTrace has the goal to develop traceability tools suitable at both the species and population levels to be applied in the monitoring of four bio-economically important fishery resources in the European Union: Atlantic cod (*Gadus morhua*), herring (*Clupea harengus*), European hake (*Merluccius merluccius*) and common sole (*Solea solea*). Preliminary findings have revealed that in the Mediterranean two flatfish species are exploited under the commercial name of common sole, *Solea solea* (the true common sole) and *Solea aegyptiaca* (the Egyptian sole).

In the Mediterranean basin, the common and Egyptian soles are two of the most valuable flatfish fishery resources, and in the past *S.aegyptiaca* was erroneously synonymised with *Solea solea*. However, recently fish biologists have proven species distinctiveness, even though the two taxa are greatly similar for rough morphology (i.e. cryptic species). The common and Egyptian sole seems to co-occur in several areas of the basin forming apparently sympatric demes. The sympatric distribution and the close phylogenetic relationship between the two sole species will allow a scenario for potential ecological and evolutionary interactions.

Although morphological methods are very useful in fish taxonomy and fishery biology, in the case of cryptic species or in species with a large plasticity of morphological and meristic traits due to convergent selection of phenotype, they suffer of lack of power (Fisher et al., 2000). Until recently, Mitochondrial DNA (mtDNA) loci have been considered the most suitable and universal markers for taxonomical questions (Avise 2004; Ratnasingham and Hebert, 2007). However, codominant nuclear DNA (nDNA) loci have several advantages over maternally inherited and haploid mtDNA, such as the capacity to detect biparentally inherited polymorphisms and recent/past species admixture (Buonaccorsi et al. 2001, Ludwig et al. 2003).

This thesis aims to advance in the taxonomic, zoogeographic, ecological and evolutionary knowledge on the Mediterranean soles i) by developing a multiplex PCR test for the rapid screening of the two cryptic species, ii) by analysing the species composition of several geographical demes and iii) the possible occurrence of interspecific hybridization and/or allele introgression in mixed populations, using

single-locus and multi-locus genetic assignment tests based on nuclear codominant markers as internal transcribed spacer of ribosomal DNA genes and microsatellite loci, respectively.

Unravelling ecological and reproductive interactions between *Solea solea* and *Solea aegyptiaca* is downstream relevant for improving the resource conservation and management and for advanced understanding of evolutionary biology of marine fish. Human activities increase the frequency of ecological relationships between populations of different species as well as between wild and domesticated individuals, and they might play significant role in the natural process of adaptation, local extinction/recolonization events, hybridization or disrupting natural selection effects. The highlighting of such ecological and genetic interactions between sole species in the Mediterranean basin enables the understanding of processes such as population divergence, speciation and hybridization that can create evolutionary novelty.

3. MATERIALS AND METHODS

3.1. Sampling

Within the FishPopTrace sampling task, sole tissue samples (N = 179) were collected from four sites (Fig. 3.1) in 2009, using commercial vessels:

- Viareggio, North Thyrrenian Sea (FAO fishery sub-area 37.1.3 – Sardinia; N = 51)
- Lagoons of Cagliari, South Sardinia (FAO fishery sub-area 37.1.3 – Sardinia; N = 49)
- Akdeniz and Antalya, Turkish coast (FAO fishery sub-area 37.3.2 – Levant; N = 21)
- Alexandria, Egypt (FAO fishery sub-area 37.3.2 – Levant; N = 58).

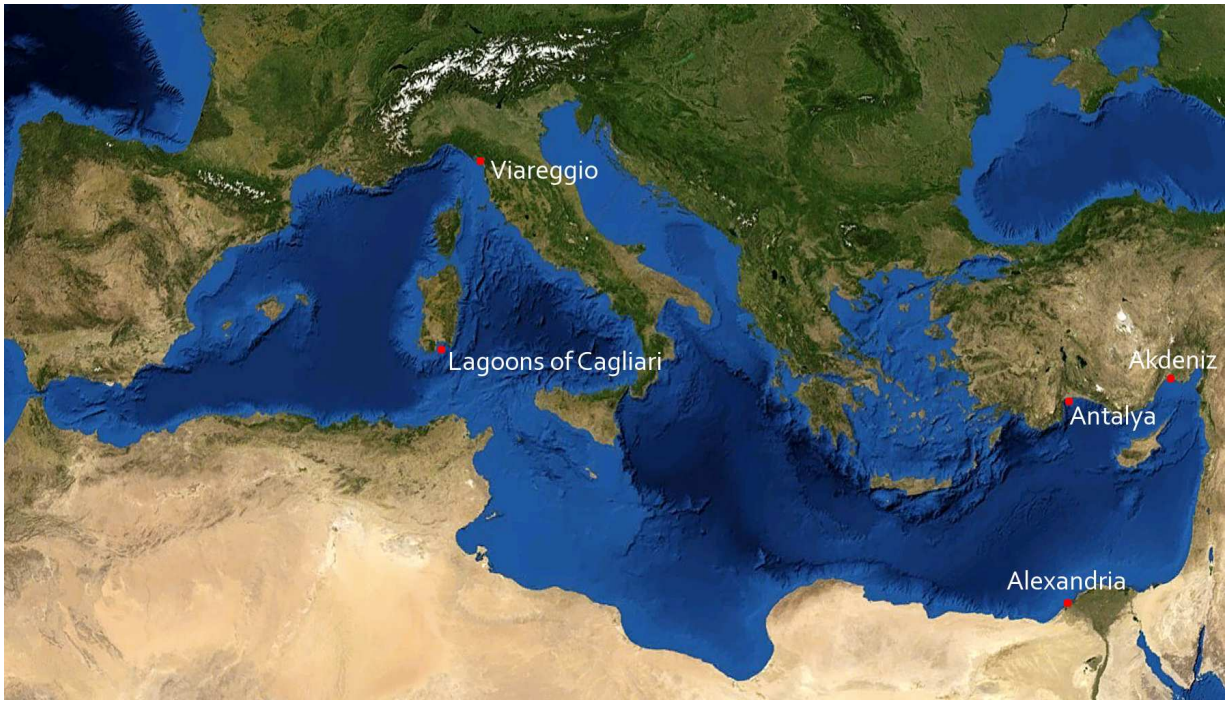


Fig 3.1: Sampling sites of common and Egyptian soles in the Mediterranean. The samples from Antalya and Akdeniz were merged in a unique sample.

These samples were previously analysed for haplotype sequence variation of the cytochrome b (cytb) mtDNA gene fragment (MSc research work of Silvia Micheli, 2011). The cytb results revealed that population samples from Lagoons of Cagliari and Turkish coasts were composed by admixture of *S. solea* and *S. aegyptiaca*, while Viareggio and Alexandria were pure populations of *Solea solea* and *S. aegyptiaca*, respectively.

3.2. Lab methods

3.2.1. DNA extraction

Individual tissues (white muscle or finclip) were stored in ethanol 96% at -20°C. Genomic DNA was extracted from ~20 mg of tissue using the CTAB-proteinase K procedure (Winnepenninckx et al. 1993). A 2 µL-aliquot of the extracted DNA solution was electrophoresed on a 0.8% agarose gel to control quality and quantity.

3.2.2. Polymerase Chain Reaction (PCR)

Here I've used both uniplex and multiplex PCR techniques; the basic difference between these two methods is that, in the uniplex PCR only one pair of primers is used whereas in the multiplex PCR from two to several primer pairs are included in the same reaction to amplify different DNA fragments. The PCR (Mullis et al., 1987) is an in-vitro enzymatic replication of the DNA. Generally, the reaction is carried out in a volume of 10–200 µL in a thermal cycler, an equipment that alternately heats and cools the reaction mixture following pre-defined steps at different temperatures and times corresponding to a denaturation step at 90-96°C, the primer annealing at ~40-60°C, and the extension/elongation phase at 72°C. These steps are repeated in a cycle for several times. The temperature and the duration of steps depend on a variety of parameters, including the enzyme used for DNA synthesis, the efficiency of primer annealing and the primers pair specificity.

3.2.3. Gel and capillary electrophoresis

Gel electrophoresis enables the separation of nucleic acids (DNA and RNA) and proteins, based on size and charge, using an electric field applied to a gel matrix. The separation of macromolecules is performed by their migration in a gel of agarose. Negatively charged molecules (as DNA and RNA) move to the anode. The DNA fragments were visualized on a UV source by adding GelRed™ Nucleic Acid Stain (Biotium) to the agarose gel (3 µL/100mL gel). An example of the result of an electrophoresis experiment using agarose gel is reported in Figure 3.2.

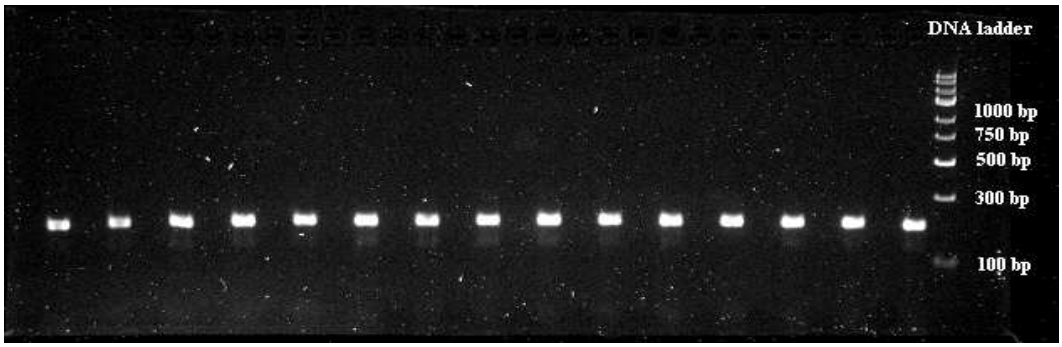


Fig. 3.2 Agarose gel showing fluorescent bands corresponding to double-stranded DNA PCR products of about 200 bp in size. In the right lane, a DNA ladder (GeneRuler™, Express DNA Ladder, Fermentas) was loaded to size approximately the DNA fragments.

Capillary electrophoresis is a family of related techniques that use narrow-bore fused-silica capillaries to perform high efficiency separations of both large and small molecules. These separations are facilitated by the use of high voltages, which may generate electroosmotic and electrophoretic flow of buffer solutions and ionic species, respectively, within the capillary. The analytes separate as they migrate due to their electrophoretic mobility, and are detected near the outlet end of the capillary thanks to their fluorescent activity. The output of the detector is sent to a data output and handling device such as an integrator or computer; the data is then displayed as an electropherogram (fig.3.3). Separated chemical compounds appear as peaks with different retention times in the electropherogram; the fragment migration time is directly related to the number of bases the fragment is composed of (http://en.wikipedia.org/wiki/Capillary_electrophoresis). The capillary electrophoresis is normally used to detect length polymorphism, as I've done in my study with the microsatellites.

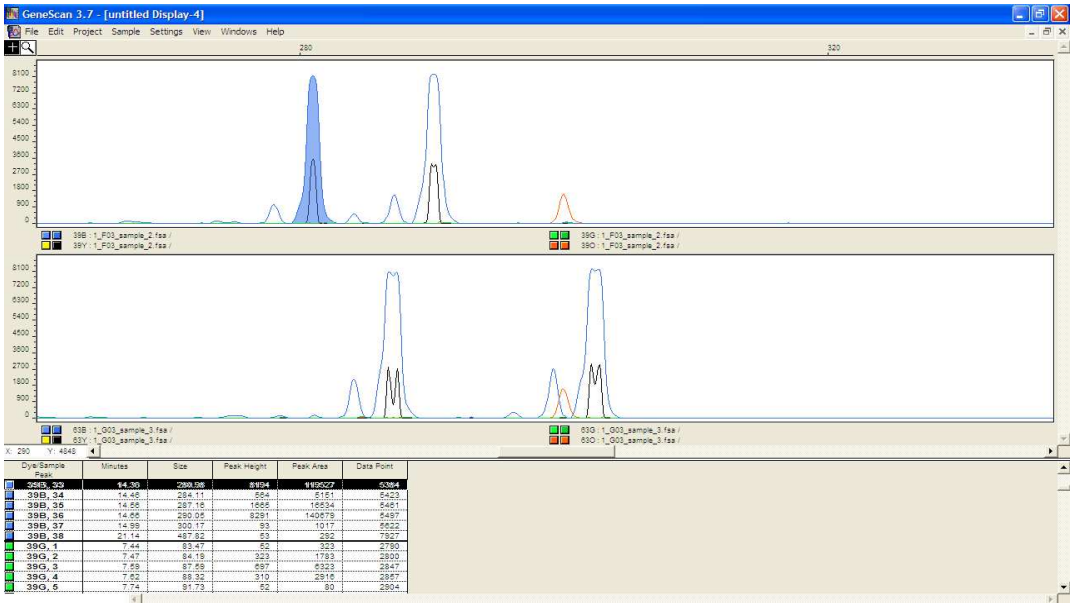


Fig. 3.3: Electropherograms of a microsatellite locus amplified in sole individuals obtained with the capillary electrophoresis technique.

Figure 3.3 illustrates the electropherogram of two individuals, both showing two-peak pattern at the locus labelled with a blue color. This pattern corresponds to a heterozygous genotype and each peak corresponds to an allele. Beside the effective allele (the higher blue peaks), PCR artefacts of amplification of microsatellites (stutter bands corresponding to smaller blue peaks) often were produced. The orange peak is a DNA fragment of known size (internal size standard).

3.3. Molecular markers and PCR conditions

Two different types of markers have been used: the microsatellites loci and the Internal Transcribed Spacer 1 of the rRNA ribosomal genes (ITS1). Primer pairs for ten microsatellite loci were available from literature (see Table 3.1) whereas ITS1 species-specific primers were newly designed for the multiplex PCR.

3.3.1. Microsatellite loci

Ten microsatellites loci were selected among those available in the literature using the following criteria:

- they should be designed for *Solea* species;
- they cross-amplify with a good yield in both target species

After a preliminary optimization work, I have chosen the loci reported in Table 3.1:

Locus name	lab code number	Reference	5' > 3' Primer sequence (F and R)	target species
F8-ICA9	1	Yyengar et al., 2000	6 FAM-ACAAGCATGCACATATG	**Common Sole**
			TTATGATTCACTGTAGC	
F8-ITG15	2	Yyengar et al., 2000	VIC-ATCATACCAAGTGTGAGACC	**Common Sole**
			GCTGATTTACTGTACTTGGC	
F13 II 8/47	3	Yyengar et al., 2000	NED-GGCTGCAGAACGATCTTTAC	**Common Sole**
			GCAACCTTGAGCTGTGACC	
F8-IGAA 7	4	Yyengar et al., 2000	NED-AGGATCTGTGGTAAATCAGC	**Common Sole**
			ACATATGTGCATGCTTGTAC	
Solga12	5	Porta and Alvarez, 2004	PET-GATCCGCTTGGGGTGAGG	**Senegalese sole**
			TGCCATACTTCACTTGTTCG	
SolA	7	Porta and Alvarez, 2004	VIC-GATCCCGACACTCACAAACG	**Senegalese sole**
			CACCCTCAGTGTAAATTGCC	
SolCa13	8	Porta and Alvarez, 2004	PET-AAGGCAGATGTCGATCACTGC	**Senegalese sole**
			TGAACAACGCCTAGAATTAGC	
ERB4	10	Eveline Diopere (KULeuven)	not available (6-FAM-)	**Common sole**
			not available	
Sos (AC)30	11	Garoia et al., 2006	NED-GTTAGGGTAAGGGGCTATGGAA	**Common Sole**
			CTACACAGCCTCATGTCTCTGG	
Sos (AC)40	12	Garoia et al., 2006	VIC-GAATGACAATACAGTAGAGACACG	**Common Sole**
			TTACCACTGAATGACTGACTGA	

Tab. 3.1: Details of the ten microsatellite loci used in this work. Primer sequences of the locus ERB4 were provided by Eveline Diopere (KULeuven) but they are not reported because still unpublished. In the thesis, loci were referred with the lab code number. All loci are fluorescently labelled (Applied Biosystem).

The loci 5, 7 and 8 were species-specifically developed for the Senegalese sole *S. senegalensis*, but they cross-amplified also in the closely related *S. solea* and *S. aegyptiaca*. The PCR thermal cycle used for all the loci except the locus 1, for which a touchdown PCR protocol was performed, is reported in Figure 3.4. The PCR conditions for the microsatellite loci are reported in Table 3.2.

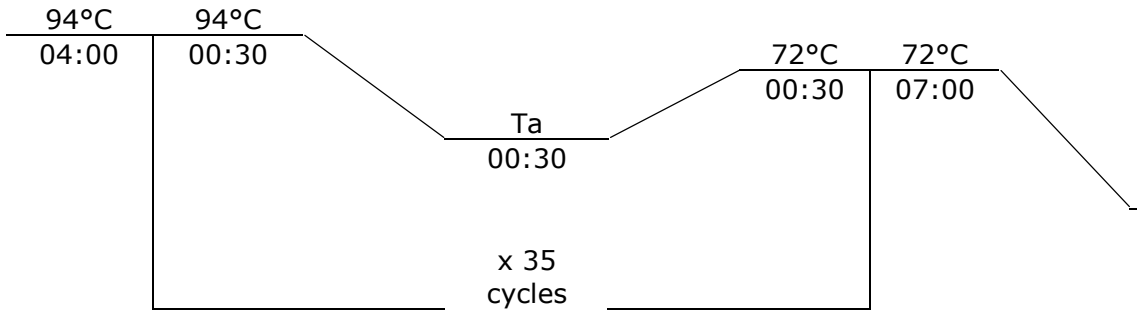


Fig.3.4: Profile of the thermal conditions used to amplify microsatellite loci 2-10.

LOCUS	Mg^{2+} (mM)	T_a (°C)	Number of cycles
1	1,5	51.5 (touchdown PCR)	10-35
2	1,5	61	35
3	1,5	61	35
4	2	59	35
5	1,5	57	30
7	1,5	59	30
8	1,2	59	30
10	1,2	54	30
11	1,5	57	35
12	1,5	58	35

Tab.3.2: Mg^{2+} final concentration, annealing temperature number of cycles used for the amplification of the microsatellite loci.

The PCR amplifications were performed in 10 µL reactions following conditions reported in table 3.2.

For the genotyping, PCR products were denaturated at 95°C for 5 min and then separated in a ABI310 Genetic Analyser. Allele sizing was carried using the LIZ 500 internal size standard (Applied Biosystem) with the GeneScan® Analysis Software (Applied Biosystem). After the initial scoring, I have discarded the loci 2 and 7 because they didn't show interpretable genotypes. Therefore, the final dataset used for the genetic variation analyses consisted of 179 individual multilocus genotypes at eight microsatellite loci.

3.3.2. ITS1

For this marker, I've developed a multiplex PCR to amplify species-specific DNA size markers in the two target sole species.

The entire Internal Transcribed Spacer 1 (ITS1) region of both target species was PCR amplified and sequenced using the primers developed by Kijewska et al. (2009) and reported in Table 3.3.

Primer name	5'-->3' sequence
ITS1 F (forward)	GTAGGTGAACCTGCGGAAGGATCATT
ITS1 R (reverse)	ATCGACGCACGAGCCGAGTGA

Tab.3.3: ITS1 primer sequences

For both species, the final concentrations of reagents in the 10 µL PCR reactions were:

- 1 X reaction buffer (Invitrogen);
- 2 mM $MgCl_2$ (Invitrogen);
- 0,5 µM primer each (Sigma);

- 0,2 mM dNTP each (Promega);
- 2% formamide;
- 0,5 U/µl TaqDNA polymerase (Invitrogen);
- 10% of template DNA (1:5 diluted solution).

The PCR conditions consisted of 30 cycles at 94°C for 1 min, T_m for 45 sec, 72°C for 45 sec. A denaturation hold at 94°C for 5 min and a elongation at 72°C for 5 min were added before and after cycling. The annealing temperature for the *S. solea* was set to 50°C, whereas that for the *S. aegyptiaca* at 52°C. The PCR products were separated by electrophoresis on a 1.5% agarose gel.

Ten PCR ITS1 fragments (~820 bp) of *S. solea* and ten of *S. aegyptiaca* were then cycle-sequenced on both strands at Macrogen Inc., Korea. Sequences obtained were edited and aligned by MEGA 4.0 (Tamura et al., 2007) software (Fig. 3.5).

The homology of the ITS1 sequences obtained was confirmed by blasting them in the GenBank (BLAST, Tatusova and Madden, 1999 NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

From the initial 20 individual sequences, I've discarded those which resulted largely

Fig.3.5: The ITS1 aligned sequence file in MEGA 4.0 (sequence window).

incomplete (<500 bp) and unreadable because of the presence of several Simple Sequence Repeats. Finally, for each species I have generated a "consensus" sequence.

Six species-specific ITS1 primers were designed using the software PRIMER3 (http://frodo.wi.mit.edu/primer3/) setting weak amplification conditions. Then, using the fastPCR 6.1 software (Kalendar et al., 2009), three multiplex PCR primer pairs were chosen for each species (Tab. 3.4) to amplify DNA fragments of different size. The reverse primers specific for *Solea solea* were paired to the primer ITS1F , whereas the forward primers specific for *Solea aegyptiaca* were paired to the primer ITS1R. All possible PCR products as well as the production of primer-dimers were tested *in silico* using fastPCR 6.1 software.

	name	Pairing primer	Lenght	Start	Product Size
Solea solea	SSr1	ITS1F	20	194	194
	SSr2	ITS1F	20	196	196
	SSr3	ITS1F	20	193	193
Solea aegyptiaca	SAf7	ITS1R	20	213	597
	SAf8	ITS1R	20	212	598
	SAf10	ITS1R	20	208	602

Tab.3.4: Multiplex ITS1 PCR primers selected for the target species.

Two ITS1 primer combinations for each species were lab tested by uniplex PCR experiments: the SSr2 and SSr3 for *S. solea* (paired to the universal ITS1F primer), and the SAf7 and SAf10 for *S. aegyptiaca* (paired to the universal ITS1R primer). After the *in vitro* tests, I selected the species-specific primer combinations SSr3-ITS1F and SAf10-ITS1R because they gave higher yields of the expected PCR products

After the uniplex PCR experiments, multiplex PCR conditions were optimized with the QIAGEN® Multiplex PCR kit, which improves the amplification yield thanks to the HotStarTaq DNA Polymerase and the Q-solution. The Q-Solution is a PCR additive reagent that facilitates amplification of difficult templates by modifying the melting behaviour of template DNA, while the HotStarTaq DNA Polymerase prevents the formation of non-specific PCR products. HotStarTaq DNA Polymerase is activated at 95°C for 15 min.

The multiplex PCR was performed in 10 µL-reactions containing:

- RNA-free water 2 µL;
- PCR Qiagen MasterMix 5 µL;
- Primer mix solution 1 µL (50 µL of Primer Mix solution consisted of 1 µL of each species-specific primer, 2 µL of each universal primer and 44 µL of TE 1X);
- Q-solution 1 µL;
- Template DNA (1:5 diluted solution) 1 µL;

The following “universal multiplex cycling protocol” proposed by the kit manufacturer was used:

- initial HotStarTaq DNA Polymerase activation step: 15 min at 95°C;
- denaturation: 30 s at 94°C;
- annealing: 90 s at 60°C;

- extension: 90 s at 72°C (from step 2 to 4 for 35 cycles);
- final extension: 10 min at 72°C.

Multiplexed PCR products were separated by electrophoresis on a 2% agarose gel and sized by loading 0.5 μL of an internal size standard (GeneRuler™ Express DNA Ladder).

3.4. Microsatellites Genetic data analysis

The complete dataset includes 179 specimens, belonging to 4 population samples (see paragraph 3.1) genotyped at the 8 microsatellites loci. A subset of 125 individuals (54 *S. solea*, 71 *S. aegyptiaca*) evenly distributed among the four population samples, was validated for species identification by the cytb haplotype data (Micheli, 2011). For each population sample, allele frequencies, number of alleles, allelic range, expected (He) and observed (Ho) heterozigosities per locus were calculated using the software GENETIX v. 4.05 (Belkhir et al., 1999). For each species, mean and single-population estimates of allelic richness per locus (A_r) were obtained with the software FSTAT 2.9.3.2 (Goudet, 2001). The software GENETIX 4.05 was also used to calculate single-locus F_{st} value, to perform a factorial correspondence analysis (AFC) of multilocus genotypes and to create suitable input files for the downstream test and software.

The program package GENALEX v. 6.41 (Peakall, R., Smouse, P.E., 2006) was used to calculate allele frequencies at each locus within each species in order to detect private alleles and to plot genetic distances among taxa (species and populations) in the Principal Coordinates Analysis (PCA).

Deviations of allele frequencies from the Hardy-Weinberg (HW) equilibrium were tested, using the software GENEPOP v. 3.4 (Raymond and Rousset, 1995), only in the population samples of Viareggio for *Solea solea,* and Lagoons of Cagliari and Alexandria for *Solea aegyptiaca.* The sequential correction of Bonferroni for multiple tests (Rice, 1989) was applied on the significance value α (with α1= 0,05 and α2=0,01).

The software MICROCHECKER 2.2.3 (Van Oosterhout et al., 2004) was used to test for scoring errors, large allele dropout and null alleles in samples affected by HW disequilibrium.

Genetic differentiation among samples within species was estimated as multilocus pairwise F_{st} using the software ARLEQUIN v. 3.11 (Excoffier et al., 2005), significance was tested on 10,000 permutations for setting the significance level at 0.01.

The WHICHLOCI 1.0 software (Banks et al., 2003) was used to select the most discriminating loci for the species identification, by simulating 3 populations with N=100, 500, 1000 based on allele frequencies data for each species. Using both Whichrun assignment and the Allele Frequency Differential method, this approach ranks loci, by trial assignments with one locus at a time, in terms of efficiency for correct population assignment. Subsequent trials with increasing numbers of loci determine the minimum number of specific loci needed to attain user defined power for species assignment.

Genetic divergence among population samples of the two sole species was investigated both at the between-species level (whose datasets included samples and individuals of both species) and at the within-species level (whose dataset included samples and individuals of only one species), using the Bayesian model-based clustering algorithm implemented in the software STRUCTURE 2.3 (Pritchard *et al.*, 2000). This clustering method allows to infer the number of genetic clusters in the data without making any a priori assumptions on population structure or species identity. The software assigns individuals into a predefined number of clusters (K) which may represent putative populations or species in order to achieve HW equilibrium and linkage equilibrium. Log-likelihood values for different Ks are provided. ΔK, a measure of the second order rate of change in the likelihood between successive K values, was calculated in order to accurately detect the most pronounced genetic subdivision (Evanno et al., 2005). At the between species level was used the Admixture model, frequencies independent while at the within species level was used the Admixture model, allele frequencies correlated; Monte Carlo Markov Chain steps = 10^5, Burnin period length = 20000 steps. The analysis was performed for each $1 < K < 5$ (five iterations per K); no prior informations about the origin of samples are given in this analysis.

At the between-species level the Bayesian model-based clustering algorithm was used to assess the genetic differentiation between the two species, while, at the within-species level, it was used to assess the most likely number of groups (genetic groups) within each species. STRUCTURE 2.3 software was used also to specifically assign the individuals whose putative species was not validated by the cytb haplotype data (N=54). In this test I have used the Admixture model, allele frequencies independent (Monte Carlo Markov Chain steps = 50000; Burnin period length = 20000 steps), but also selecting the "USEPOPINFO" prior that pre-specifies the species-identity (*S. solea* or *S. aegyptiaca*) of the 125 individuals with cytb data. Therefore, these 125 individuals were

separated in two species-specific groups 1 and 2 to assist the ancestry estimation of the 54 individuals, with species ID not validated by cytb data, sorted in a third group.

The software GENECLASS 2.0 (Piry et al., 2004) was used for multilocus individual assignment test. This software computes various genetic assignment criteria to assign or exclude reference populations as the origin of diploid or haploid individuals, as well as of groups of individuals, on the basis of multilocus genotype data. In all analyses, I used a Monte-Carlo re-sampling probability computation with Rannala and Mountain (1997) Bayesian algorithm, with one thousand of simulated individuals and α error set to 0.01. The correct assignment threshold score was fixed to 0.05. The assignment of individuals to the sole species (putatively identified according to the ITS1 and cytb results when available) was carried out firstly using the entire panel of 8 microsatellite loci and secondarily, using only the loci selected by the test based on the software WHICHLOCI. The individuals of a given species were also assigned to the macro-geographical area (i.e. Eastern and Western Mediterranean), and then to the population sample to test the power of the assignment within species.

4. RESULTS

4.1. Multiplex PCR ITS1 assay

The multiplexed PCR ITS1 products were resolved on a 2% agarose gel: as expected by primer and marker design, specimens of the two species gave bands which differed in length; these differences provided two species-specific ITS1 band profiles (Fig. 4.1).

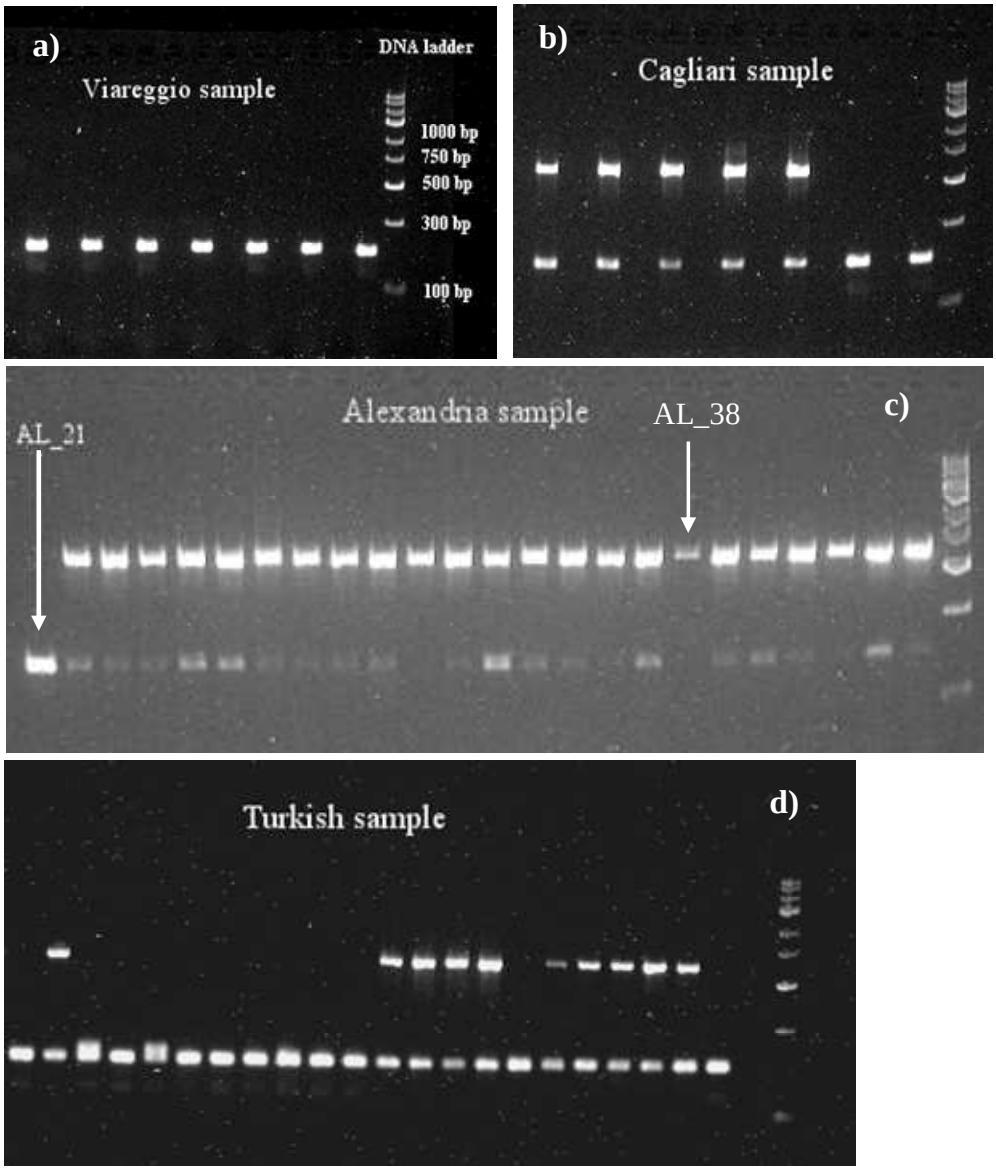


Fig. 4. 1: Agarose -gel separation of the ITS1 PCR amplicons in specimens of the four population samples. a) Viareggio, seven individuals displaying a *S. solea* multiplex PCR ITS1 profile; b) Lagoons of Cagliari, individuals displaying *S. aegyptiaca* (five individuals from the left) and *S. solea* (the last two individuals) profiles; c) Alexandria, all individuals except two displaying the *S. aegyptiaca* profile. The individual AL21 gave a *S. solea* profile while the individual AL38 gave only the *S. aegyptiaca* band. In the last lane of all gels, the DNA ladder GeneRuler™, Express DNA Ladder, Fermentas, was loaded.

The *S. solea* individuals gave the expected 193-bp band (all specimens of Fig. 4.1A) while *S. aegyptiaca* individuals gave the expected species-specific band at 602 bp. However, unexpectedly putative *S. aegyptiaca* individuals also gave a 193-bp band; therefore the species-specific PCR profile of this species was characterized by the occurrence of the two species-specific bands at 602bp and 193bp (see for example the specimens 1-5 in Fig. 4.1B). The universal ITS1 fragment didn't amplified in any individual. The failure of its amplification in the multiplex PCR is likely due to the larger size of this fragment than those of the species-specific fragments (820 bp vs. 193 bp in *S. solea* and 602 bp in *S. aegyptiaca*) that could have disadvantaged its amplification.

The multiplex amplification of both species-specific bands in the individuals putatively assigned to *S. aegyptiaca* by cytb haplotype prevented the potential discrimination of interspecific *S. aegyptiaca* × *S. solea* hybrids or gene-introgressed individuals among themselves (assuming that all *S. aegyptiaca* individuals can not be considered interspecific hybrids or gene-introgressed; see Discussion). On the contrary, any of the individuals putatively assigned to *S. solea* by cytb haplotype showed the two-band multiplex PCR ITS1 profile, ruling out the occurrence of both *S. solea* × *S. aegyptiaca* hybrids among them and individuals displaying an introgression of *S. aegyptiaca* ITS1 genes in *S. solea*.

Tab. 4.1: Summary results of the multiplex PCR ITS1 assay for species identification in the ITS1 and other two markers of the four population samples.

SAMPLES	**SAMPLE SIZE**	**SPECIES**		**MARKER**	
			ITS1	**cytb**	**STRs**
VIAREGGIO	51	SS	51	36	51
		SA	0	0	0
LAGOONS OF CAGLIARI	49	SS	8	6	8
		SA	41	18	41
ALEXANDRIA	58	SS	1	1	1
		SA	57	43	57
TURKISH COASTS	21	SS	11	11	11
		SA	10	10	10
TOTAL	**179**	SS	**71**	**54**	**71**
		SA	**108**	**71**	**108**

SS= *S. solea*; SA= *S. aegyptiaca*. The cytb validation was available only for 125 individuals, while ITS1 and STRs information were available for the whole dataset.

From the multiplex PCR ITS1 assay in the 179 individuals some outcomes are relevant to be outlined. The Table 4.1 reports the summary results of the ITS1 genotypes of the

four population samples compared with the other two markers (cytb and microsatellites).

The multiplex PCR ITS1 assay gave evidence that the Viareggio population sample is uniquely composed by *S. solea*, while in the Lagoons of Cagliari and Turkish samples the two species co-occurred. All individuals from Alexandria except two (AL21 and AL38; Fig. 4.1c) exhibited a *S. aegyptiaca* ITS1 profile. The individual AL21, provided a *S. solea* profile (confirmed also by the cytb haplotype obtained during this research work and the microsatellite genotypes) while the AL38 individual gave only the 602 bp *S. aegyptiaca* species-specific band. However, this latter individual did not amplify also at several microsatellite loci and therefore the lack of the *S. aegyptiaca* profile was likely due to unsuitable quality of the extracted genomic DNA.

4.2. Microsatellites

4.2.1. Genetic diversity

The mean estimates of the principal genetic diversity parameters at each of the microsatellite loci in the two sole species are reported in Table 4.2.

Tab. 4.2: Mean estimates of genetic diversity parameters and of genetic differentiation indexes between the two sole species at the eight microsatellite loci.

	LOCUS 1		LOCUS 3		LOCUS 4		LOCUS 5	
	SS	SA	SS	SA	SS	SA	SS	SA
N	71	107	69	102	71	104	66	103
N_a	6	6	11	6	6	3	18	6
Allelic Range	87-103	79-89	163-191	165-183	126-141	132-165	73-123	57-87
A_r	5.991	5.953	10.845	6	5.883	2.981	17.816	5.99
H_e	0.6919	0.5818	0.5561	0.4917	0.3336	0.0836	0.8236	0.7586
H_o	0.7606	0.4299	0.5652	0.2745	0.338	0.0769	0.7424	0.3883
# private alleles	4	4	7	2	4	1	16	4
Single-locus F_{st}	0.3239**		0.0035		0.8086**		0.2063**	
	LOCUS 8		LOCUS 10		LOCUS 11		LOCUS 12	
	SS	SA	SS	SA	SS	SA	SS	SA
N	66	102	67	102	69	107	64	107
N_a	11	3	14	6	18	11	12	2
Allelic Range	167-203	170-182	266-311	239-275	139-177	145-177	172-194	164-166
A_r	10.877	3	13.948	6	17.699	10.906	12	1.953
H_e	0.5786	0.139	0.8552	0.5708	0.8504	0.7136	0.7938	0.0093
H_o	0.4848	0.1471	0.8657	0.4902	0.7536	0.4953	0.7031	0.0093
# private alleles	9	1	11	3	8	1	12	2
Single-locus F_{st}	0.6353**		0.2860**		0.1931**		0.6646**	
Multilocus F_{st}	0.4082**							

Species: SS= *S. solea*; SA= *S. aegyptiaca*; N: sample size;
Genetic diversity parameters: N_a: allele number; A_r: allelic richness; H_e: expected heterozigosity; H_o: observed heterozigosity
Genetic differentiation indexes: # private alleles: number of private alleles; Single-locus F_{st} and Multilocus F_{st}
Significance level: * $p < 0.01$; ** $p < 0.001$

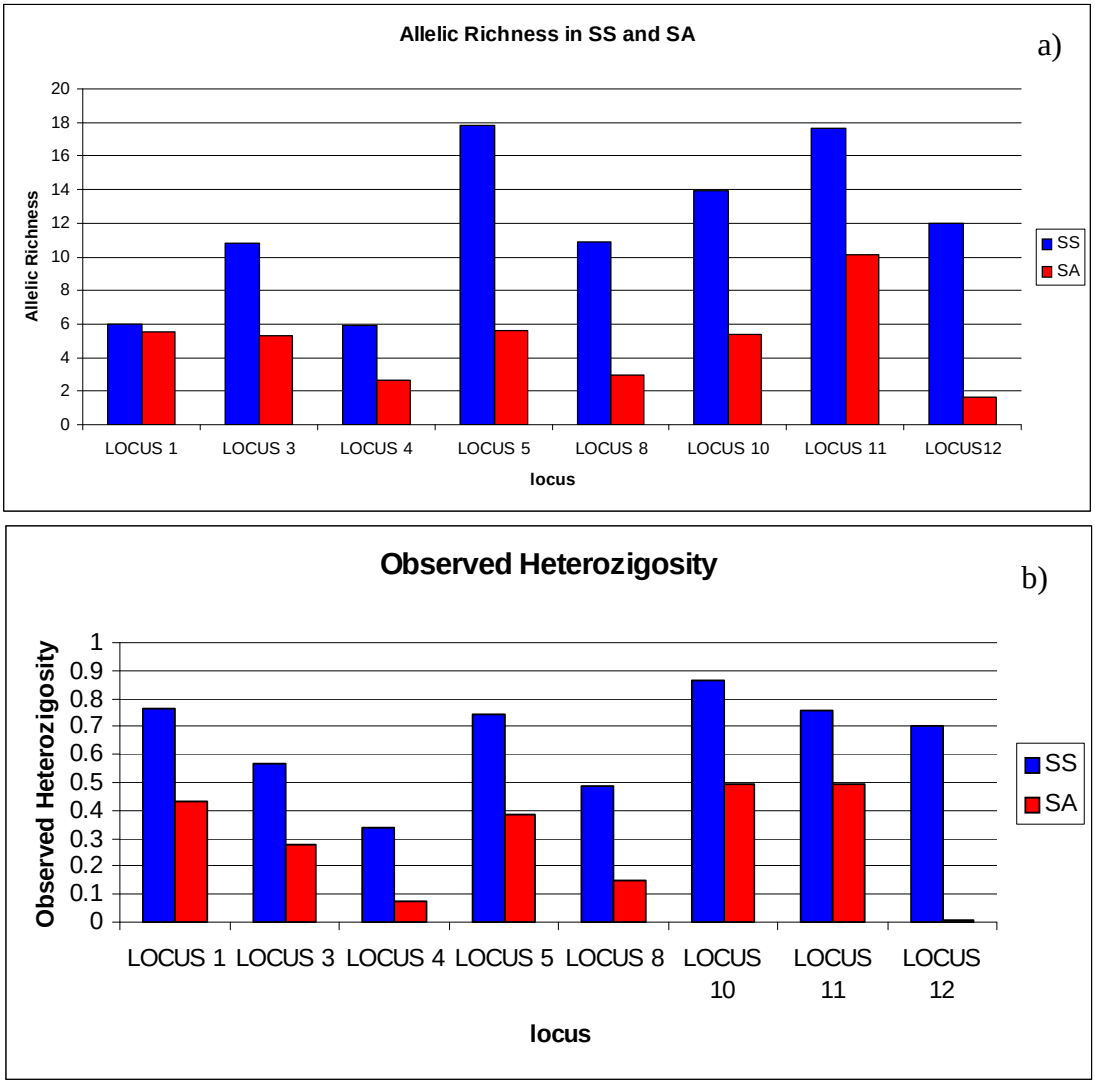


Fig. 4.2: Comparison of the allelic richness (a) and observed heterozigosity (b) mean values at each locus between the two sole species (SS: *S. solea*; SA: *S. aegyptiaca*)

Almost all mean estimates of genetic diversity resulted higher in *S. solea* than in *S. aegyptiaca* at all loci, even though the dataset included 108 *S. aegyptiaca* individuals and 71 *S. solea* individuals (Tab. 4.2).

This pattern was clearly detectable in the comparisons of the allelic richness and heterozygosity mean values (Fig. 4.2). In *S. solea*, the loci 3, 5, 10, 11 and 12 were the most variable, whereas in *S. aegyptiaca* the most polymorphic loci were 1, 3, 5, 10, and 11 (Table 4.2 and Fig. 4.2)

The two species displayed different distributions of allele frequencies at all loci (Fig. 4.3) except the locus 3, in which *S. solea* and *S. aegyptiaca* shared the most frequent allele. On the contrary, the two species did not share any allele at the locus 12 (Figure 4.3; tab 4.2). Private alleles of both sole species have been found at all loci, though their

number was quite variable across species and loci (Table 4.2). The *S. solea* showed a greater number of private alleles than *S. aegyptiaca* and at the loci 5, 10 and 12 *S. solea* exhibited exceptional numbers of private alleles (> 10).

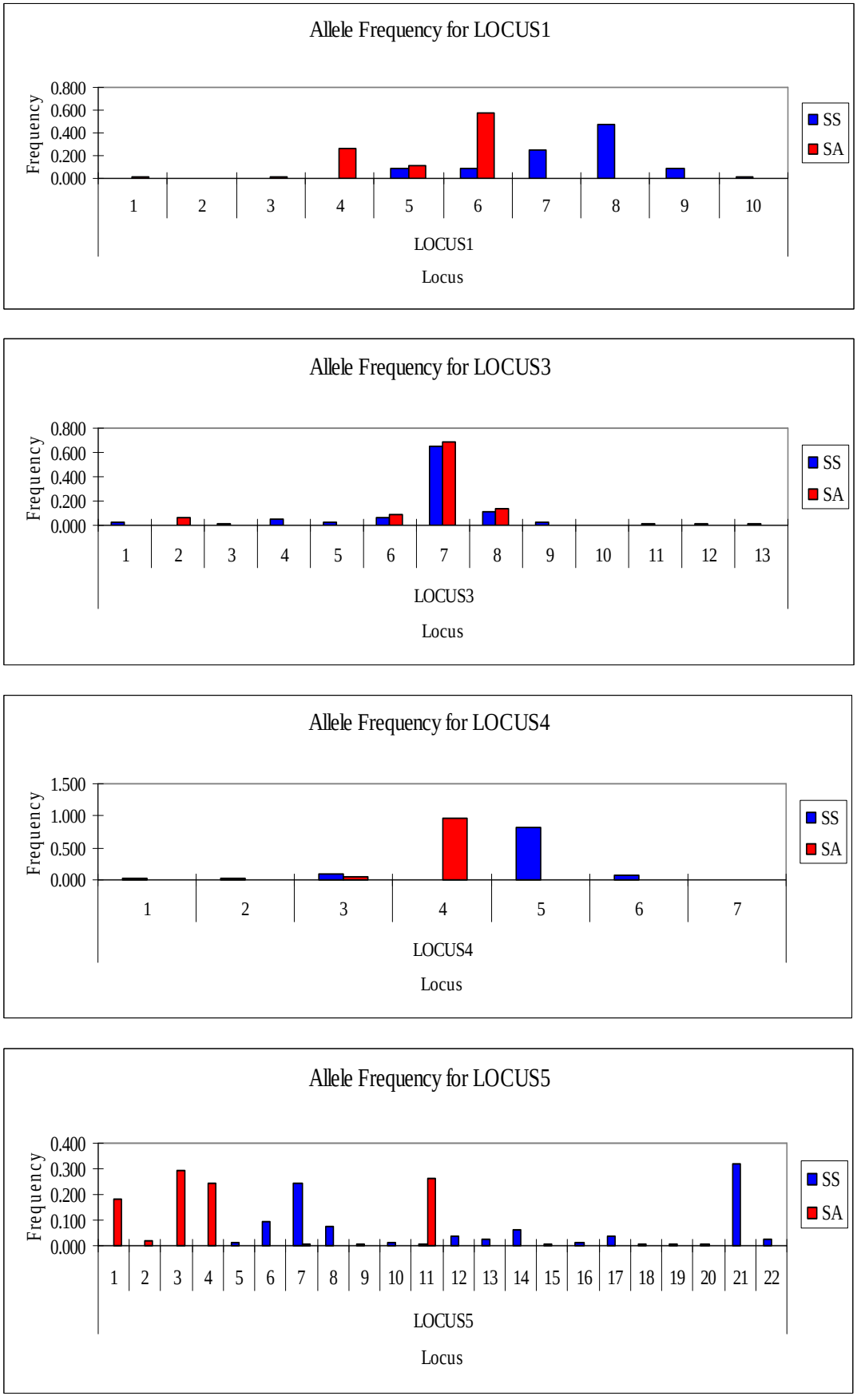
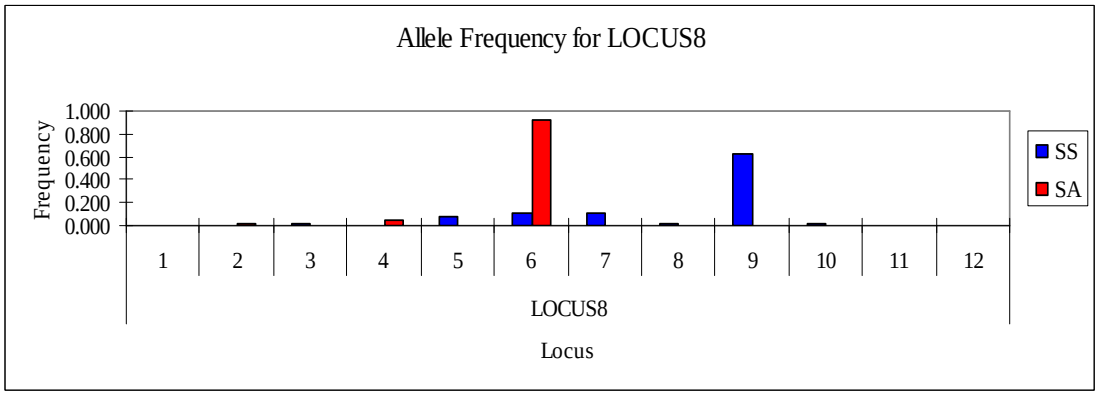
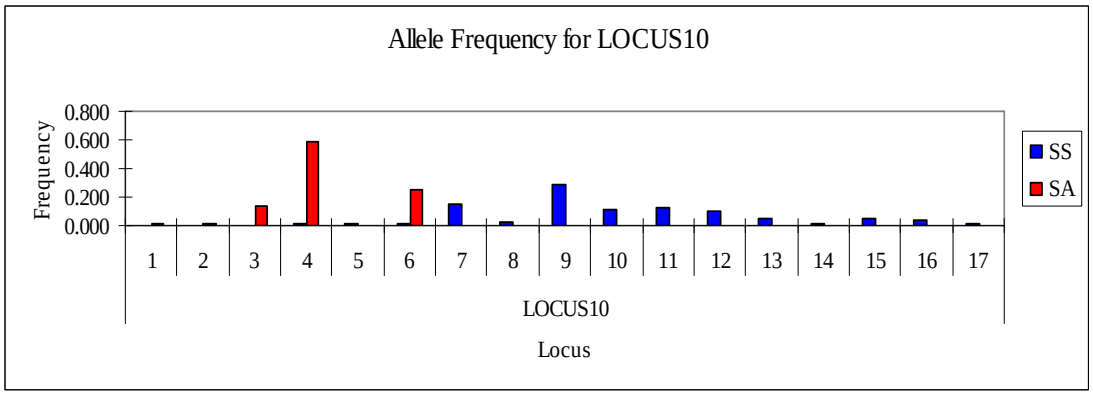
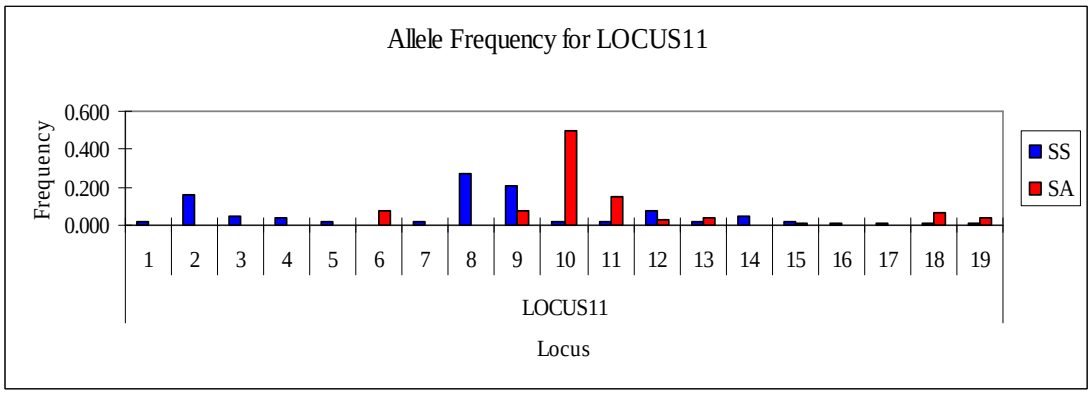


Fig.4.3: Comparison of the allele frequency distributions at the eight microsatellite loci in the two sole species *S. solea* (SS) and *S. aegyptiaca* (SA).







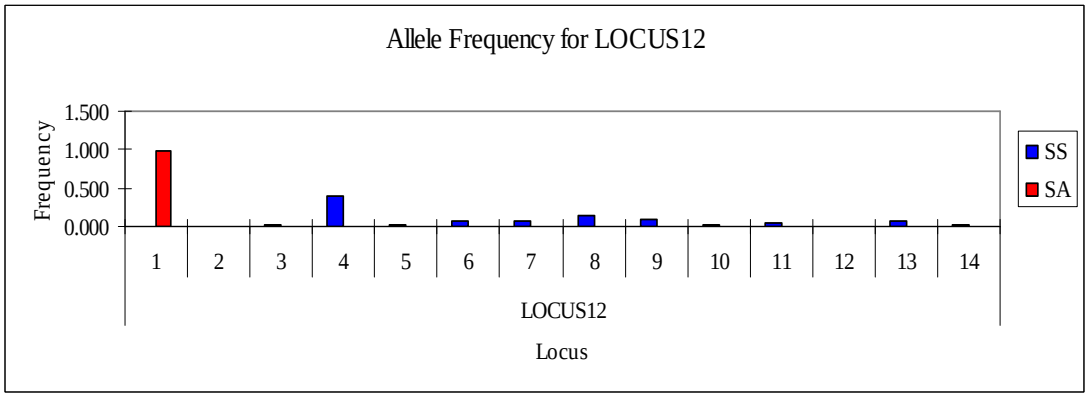


Fig.4.3: -continued - Comparison of the allele frequency distributions at the eight microsatellite loci in the two sole species *S. solea* (SS) and *S. aegyptiaca* (SA).

The estimates of the genetic diversity parameters at each locus in the population samples of the two sole species are reported in the Tables 4.3 and 4.3bis. The unique *S. solea* individual found in the Alexandria sample was not considered in this analysis and therefore omitted in Table 4.3.

Tab.4.3: Estimates of genetic diversity parameters and of genetic differentiation indexes in the population samples of *S. solea* at the eight microsatellite loci

S. solea	LOCUS 1	LOCUS 3	LOCUS 4	LOCUS 5	LOCUS 8	LOCUS 10	LOCUS 11	LOCUS 12
Viareggio								
N	51	49	51	48	48	50	50	46
N_a	6	9	5	18	10	12	16	10
Allelic range	87-103	163-189	126-141	73-123	167-203	266-311	141-177	172-192
A_r	4.009	3.316	2.387	5.963	3.998	5.925	5.485	5.191
H_e	0.7131	0.4896	0.3135	0.8533	0.6296	0.86	0.8275	0.8036
H_o	0.8039	0.4898	0.3333	0.8542	0.5625	0.90	0.76	0.7391
Lagoons of Cagliari								
N	8	8	8	6	7	5	8	6
N_a	5	6	5	5	4	5	9	6
Allelic range	87-99	163-185	126-141	79-99	179-191	275-296	141-163	172-190
A_r	4.089	4.742	4.000	4.500	3.857	5.000	7.214	5.318
H_e	0.6667	0.7667	0.6083	0.7273	0.7143	0.7556	0.9333	0.7576
H_o	0.75	1	0.5	0.6667	0.4286	0.6	0.75	0.6667
Turkish coasts								
N	11	11	11	11	10	11	10	11
N_a	4	6	2	5	3	7	7	5
Allelic range	89-99	167-191	132-138	79-123	176-191	269-287	139-165	174-194
A_r	3.504	3.675	1.714	3.078	2.000	5.555	5.303	3.853
H_e	0.619	0.5083	0.1732	0.4069	0.1947	0.8571	0.8421	0.6623
H_o	0.5455	0.5325	0.1818	0.3636	0.2	0.8182	0.7	0.5455
Single-locus Fst	0.0049	0.1312**	0.0216	0.1542**	0.0412	0.0155	0.0161	0.0343
Multilocus Fst	0.0535**							

N: sample size; N_a: allele number; A_r: allelic richness; H_e: expected heterozygosity; H_o: observed heterozygosity.
F_{st} Significance level: * $p < 0.01$; ** $p < 0.001$
Deviation from Hardy-Weinberg equilibrium significance level: * $p < 0.05$; ** $p < 0.01$ reported in the H_o field.

Tab.4.3 bis: Estimates of genetic diversity parameters and of genetic differentiation indexes in the population samples of *S. aegyptiaca* at the eight microsatellite loci

S. aegyptiaca	LOCUS 1	LOCUS 3	LOCUS 4	LOCUS 5	LOCUS 8	LOCUS 10	LOCUS 11	LOCUS 12
Lagoons of Cagliari								
N	41	37	41	38	38	38	40	41
N_a	5	6	3	4	3	3	6	1
Allelic range	79-89	165-183	132-165	69-87	170-182	260-272	149-163	164
A_r	3.642	4.335	2.095	3.049	1.798	2.761	4.049	1.000
H_e	0.6188	0.6927	0.2002	0.5049	0.1021	0.3407	0.6747	0
H_o	0.5122	0.5946	0.1951	0.1842**	0.1053	0.2895	0.65	0
Alexandria								
N	56	55	54	55	55	55	57	56
N_a	3	3	1	4	3	6	11	2
Allelic range	85-89	173-177	135	57-87	170-182	239-275	145-177	164-166
A_r	2.935	2.060	1.000	3.513	2.010	3.746	6.512	1.161
H_e	0.4921	0.2123	0	0.6901	0.1833	0.6477	0.75	0.0179
H_o	0.4286	0.0182**	0	0.5818	0.2	0.6545	0.4737**	0.0179
Turkish coasts								
N	10	10	9	10	9	9	10	10
N_a	2	2	1	2	1	2	1	1
Allelic range	83-89	175-177	135	69-87	182	266-272	157	164
A_r	2.000	2.000	1.000	1.900	1.000	2.000	1.000	1.000
H_e	0.2684	0.5211	0	0.1	0	0.4248	0	0
H_o	0.1	0.5	0	0.1	0	0.3333	0	0
Single-locus Fst	0.1678**	0.2373**	0.0893*	0.3689**	0.0205	0.1619**	0.1336**	0.0091
Multilocus Fst	0.2114**							

N: sample size; N_a: allele number; A_r: allelic richness; H_e: expected heterozygosity; H_o: observed heterozygosity.
F_{st} significance level: * $p < 0.01$; ** $p < 0.001$
Deviation from Hardy-Weinberg equilibrium significance level: * $p < 0.05$; ** $p < 0.01$ reported in the H_o field.

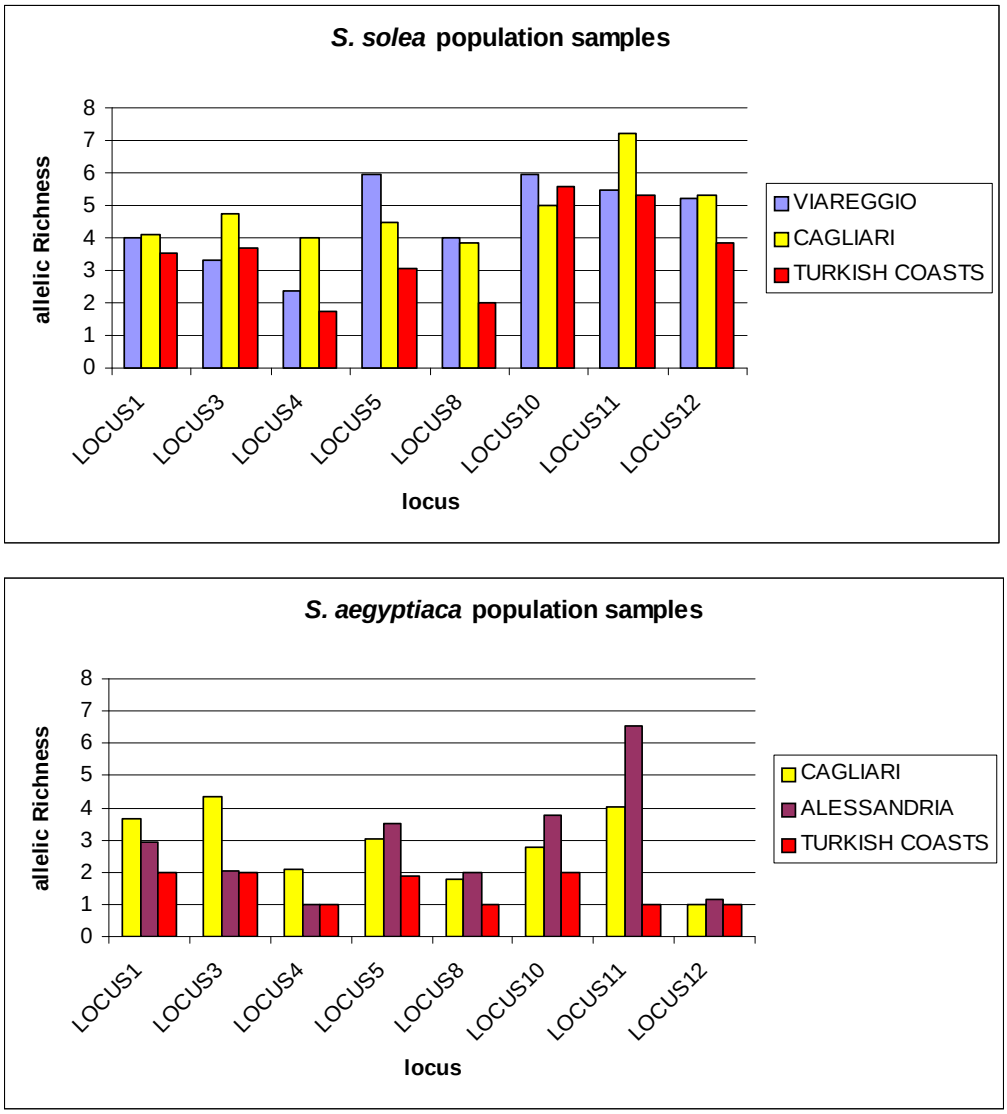


Fig. 4.4: Allelic richness of population samples of *S. solea* (SS) and *S. aegyptiaca* (SA).

Within *S. solea* (Table 4.3), genetic diversity parameters were generally high and greatly similar across population samples. However, at some loci some population samples displayed reduced polymorphism level (locus 4, Viareggio and Turkish coasts; locus 8, Turkish coasts). The allelic richness estimates were quite homogeneous across samples at a given locus (Fig. 4.4). In *S. aegyptiaca*, the lower level of genetic polymorphism (already observed at the specie-level; Table 4.2) was apparent at the population level. The three samples showed a general pattern of lack or marked reduction of genetic diversity at several loci (Table 4.3bis). The absence/reduction of genetic polymorphism was more pronounced in the samples from the Eastern Mediterranean (Alexandria and Turkish coasts). In these samples, the genetic polymorphism level did not seem related to the low sample size because Alexandria (N

> 50 at each locus) showed the lack of variation at the locus 4 and a reduced variation at the loci 3, 8 and 12 (Table 4.3bis; Fig. 4.4). The deviations from the HW equilibrium, were tested only in the population samples of Viareggio for *S. solea*, and of Lagoons of Cagliari and Alexandria (after excluding the AL21 *S. solea* specimen) for *S. aegyptiaca*, because these were statistically representative samples (N > 40). While the Viareggio *S. solea* sample was at HW equilibrium at all the 8 loci, the Alexandria sample showed significant deviations at loci 3 and 11, and Lagoons of Cagliari *S. aegyptiaca* sample showed a significant deviation at locus 5 (Table 4.3bis). Furthermore, the MICROCHECKER test revealed that these loci might be affected by null allele artefacts as suggested by the general excess of homozygotes, or stutter bands causing scoring errors.

4.2.2. Genetic differentiation between sole species

Genetic differentiation between the two species was high and significant, (multilocus F_{st} = 0.4082, $p < 0.001$; Table 4.2). Almost all microsatellite loci contributed relevantly to the genetic differentiation of the two species, being all single-locus F_{st} values high and significant except that at the locus 3 (Table 4.2). The factorial correspondence analysis of the individual multilocus genotypes grouped all specimens into two well-distinct clusters corresponding to the two *Solea* species (Fig.4.5).

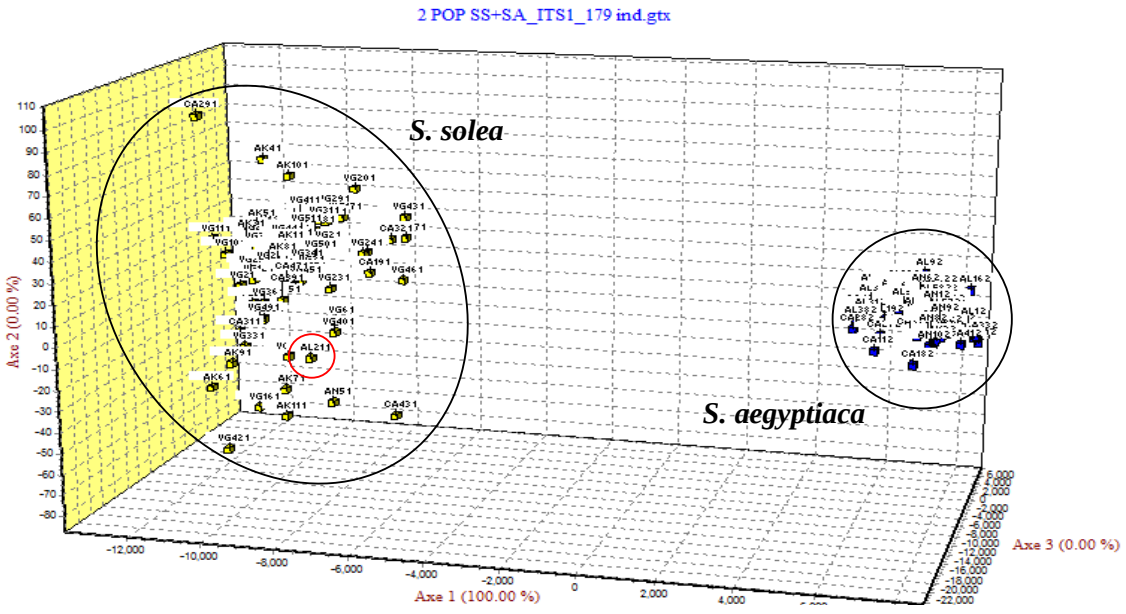


Fig.4.5: Factorial correspondence analysis of the 179 sole individuals based on 8 microsatellite loci. The first axe explains 100% of the variability, showing the complete genetic distinction between the two species

The AL21 specimen from Alexandria (red circled in the Fig. 4.5) is unequivocally grouped to the *S. solea* cluster. This result is fully consistent with the results of the multiplex PCR ITS1 test (see paragraph 4.1) and with the cytb haplotype (see Table 4.1 and Appendix 1).

Genetic differentiation between sole species was also analysed with the Bayesian clustering approach implemented in STRUCTURE (see paragraph 3.4) using two different datasets. The first analysis was carried out on a dataset including only the individuals with species ID confirmed by ITS1 and cytb data (N=125, 54 *S. solea* and 71 *S. aegyptiaca*). The second STRUCTURE analysis was carried out using the whole dataset (N = 179, 71 *S. solea* and 108 *S. aegyptiaca*). The results of these analyses are reported in Figures 4.6 and 4.7, respectively. The software represents the identified clusters (K) through a bar plot, where in x-axis each bar represents one individual and the y-axis display the percentage value of membership to a defined cluster for each individual. The output gives back also the log-Likelihood values for the different Ks, and ΔK, that indicates the most reliable K depending on the Likelihood between successive K.

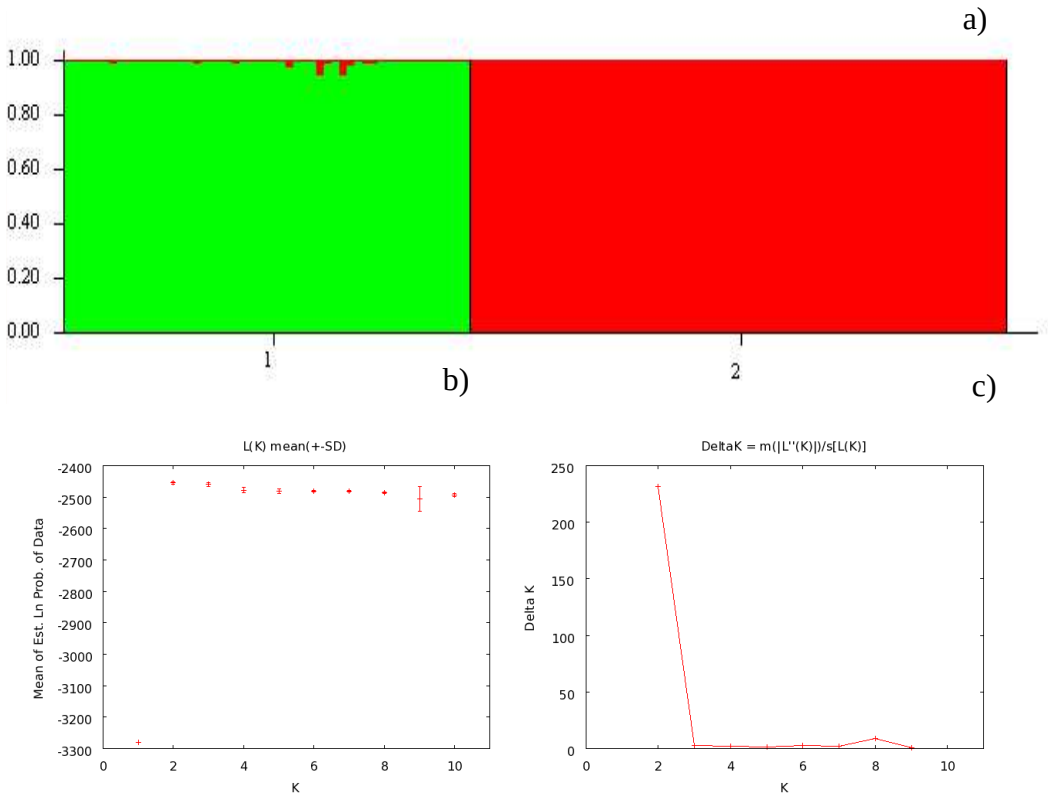


Fig. 4.6: Results of STRUCTURE clustering for the subset of individuals: **a)** K=2; **b)** log-Likelihood values for different K and **c)** ΔK.

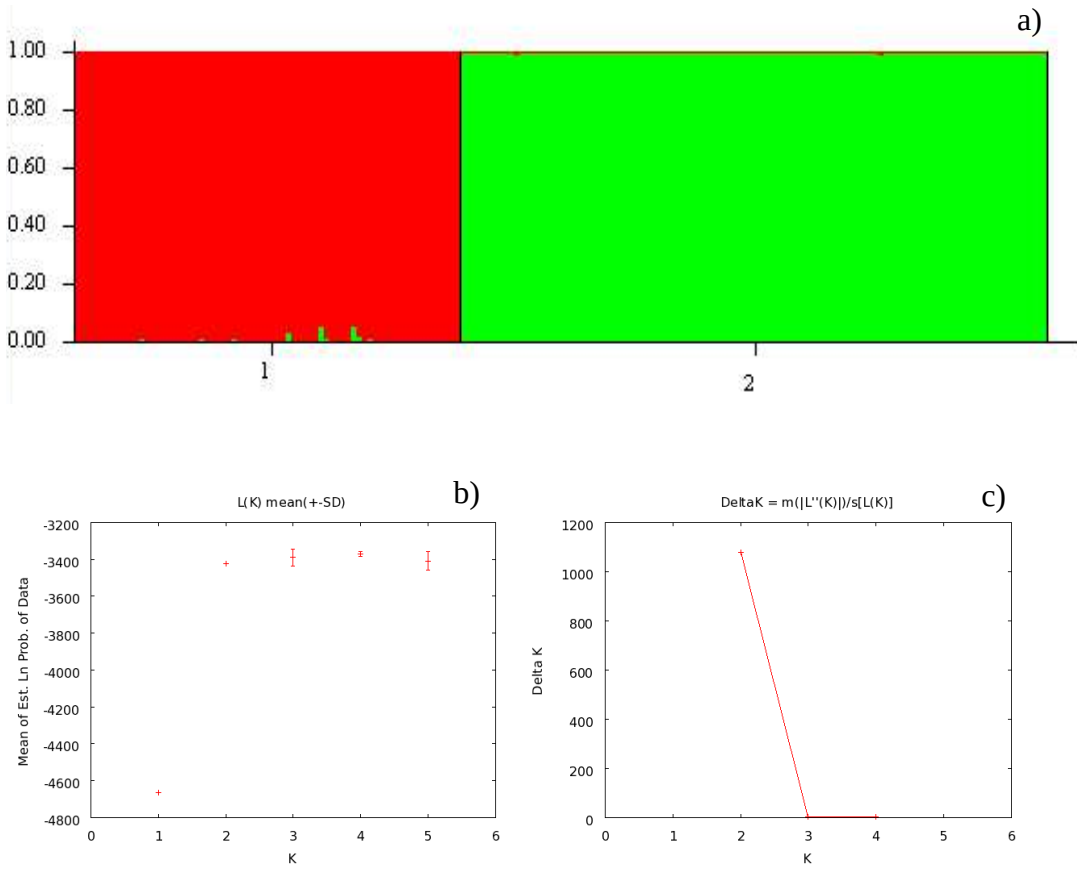


Fig. 4.7: Results of STRUCTURE clustering of the whole dataset: **a)** K=2; **b)** log-Likelihood values for different K and **c)** ΔK

At the between-species level, both STRUCTURE analyses (Fig. 4.6a and Fig. 4.7a) revealed two well-distinct clusters of individuals corresponding to *S. solea* (cluster 1) and to *S. aegyptiaca* (cluster 2) individuals, without significant differences between the results obtained with the two datasets.

After that, I have carried out a STRUCTURE analysis on the whole dataset, giving the prior information of species-assignment (selecting the USEPOPINFO prior Group 1 = *S. solea*; Group 2 = *S. aegyptiaca*) to the 125 individuals with the species identification validated by the cytb haplotype data. The remaining 54 individuals (for which the cytb haplotype data were lacking) were assigned to a generic Group 3 (namely without a pre-defined species assignment). Within this Group 3, 54 individuals were assigned to cluster 1 and 71 to the cluster 2, corresponding to *S. solea* and *S. aegyptiaca*, respectively. The results of this analysis are displayed in Fig. 4.8.

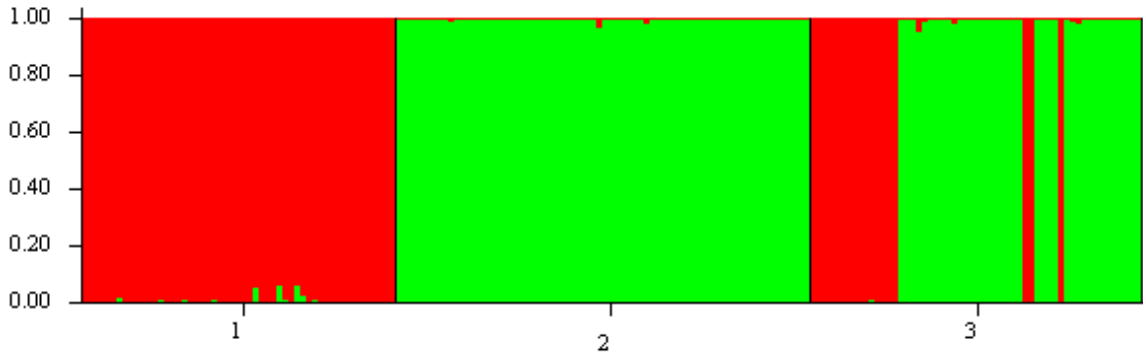


Fig. 4.8: Results of clustering analysis using the POPINFO prior information. Individuals of sample 3 (each represented by a single bar in the plot) were assigned to one of the two pre-defined species clusters.

The individual bar plot (a plot option given by STRUCTURE software) of each individual is consistent with the species ID based on the multiplex PCR ITS1 test (data not shown). It is important to note that, in all analyses, any individual did show intermediate bar plot (namely interspecific hybrid genotypes) once for all excluding the occurrence of past and present interspecific hybridization events, at least in these population samples.

Finally, the multilocus individual assignment test, performed with GENECLASS v. 2.0, also confirmed the results illustrated since now. The sole individuals were correctly assigned to the species putatively defined by the multilocus cytb-ITS1 genotype (N = 125) or by the ITS1 genotype. The assignment test performed using only the locus 12 (selected for the highest discriminative power through WHICHLOCI software) gave a ~100% of correct species assignments (data not shown). This is due to the completely different allelic ranges of the two sole species at this locus (see Table 4.2 and Fig. 4.3). Figure 4.9 illustrates the values of correct assignment obtained with GENECLASS 2.0, considering individuals assigned to i) species (bar plots 1 and 2), ii) species and Mediterranean basin (bar plots 3-6), and iii) species and populations (bar plots 7-12).
All test provided percentages of correct assignment over 85%; only the *S. solea* sample of Cagliari has a lower self assignment value, but this could depend on the small size of the sample (N=8).

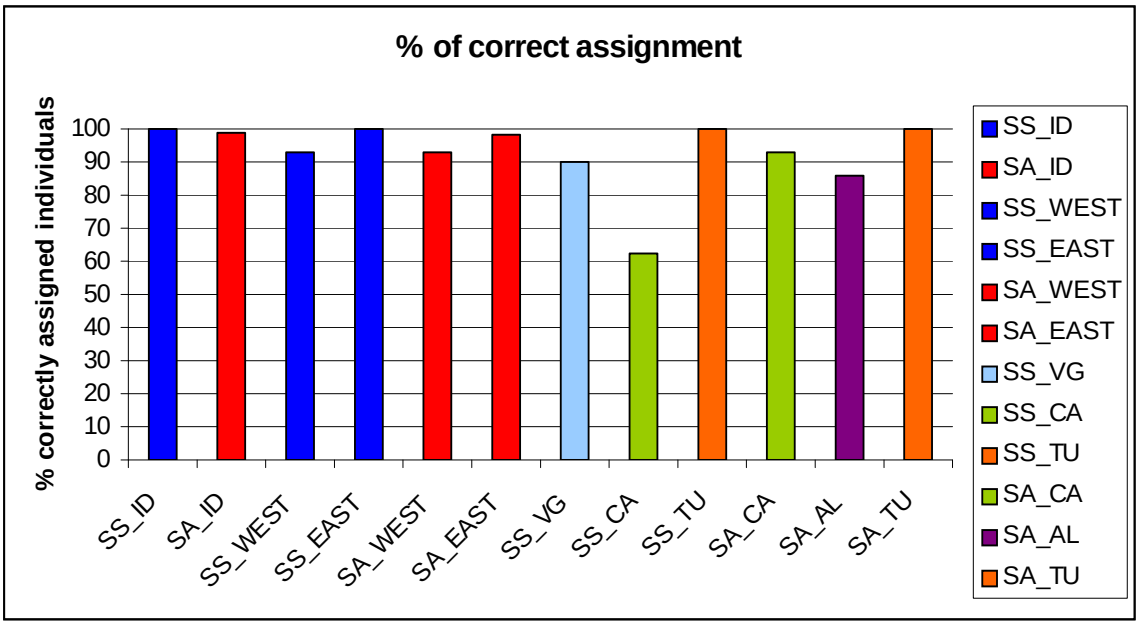


Fig. 4.9: Summary of the results of the assignment test carried out with GENECLASS.

4.2.3. Genetic differentiation within sole species.

Genetic differentiation within *Solea* species was initially surveyed through a Principal Components Analysis (PCA) whose results are illustrated in Figure 4.10.

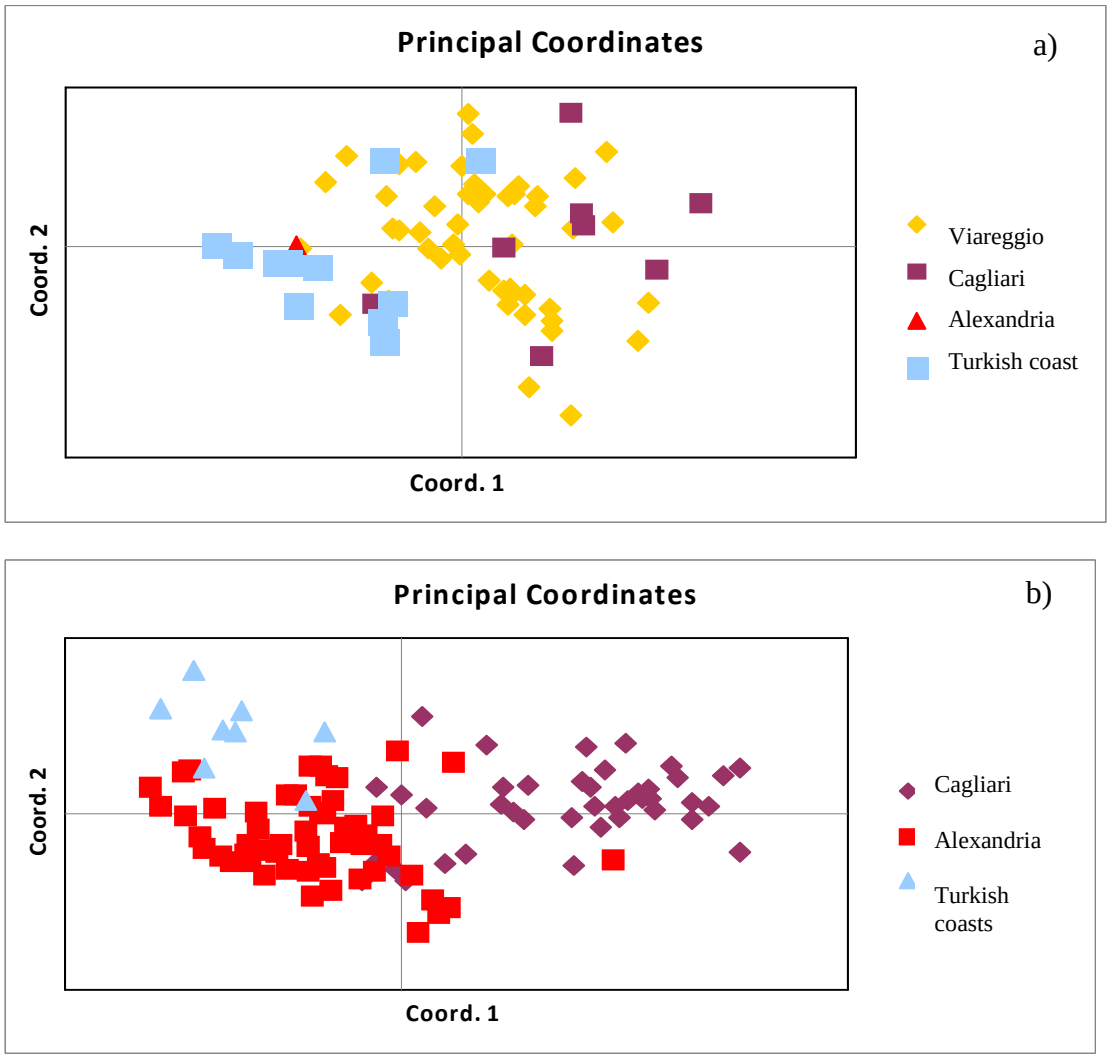


Fig.4.10: Principal Component Analyses of *S. solea* (a) and *S. aegyptiaca* (b) individuals

In *S. solea*, the PCA plot did not detect a clear spatial differentiation among population samples (Fig. 4.10a): the first coordinate explains 23% of variation, whereas the second and third coordinates explain 20% and 18% of variation, respectively. Significant F_{st} values were observed at only two loci and in the multilocus estimate (Tab. 4.3), indicating an overall low but significant level of differentiation among *S. solea* populations.

The AL21 *S. solea* specimen from Alexandria (represented in the Fig. 4.10a by a red triangle) is grouped by PCA within the Turkish individuals. This finding could suggest that this individual could be a putative migrant from Turkish coasts.

In *S. aegyptiaca*, the PCA plot (fig.4.10b) exhibited a greater power to spatially separate the individuals of the Eastern Mediterranean form those of the Western Mediterranean. In the plot, the first coordinate explained 29% of variation, while the second and third coordinates explained 19% and 15% of variation, respectively. The genetic differentiation among *S. aegyptiaca* populations was also confirmed by the high and significant single-locus F_{st}s (observed at six out of eight loci) and multilocus F_{st} (Table 4.3bis).

After that, an assignment test of individuals to putative populations was performed using the software STRUCTURE. The results of this analysis are reported in Figures 4.11 (*S. solea*) and 4.12 (*S. aegyptiaca*).

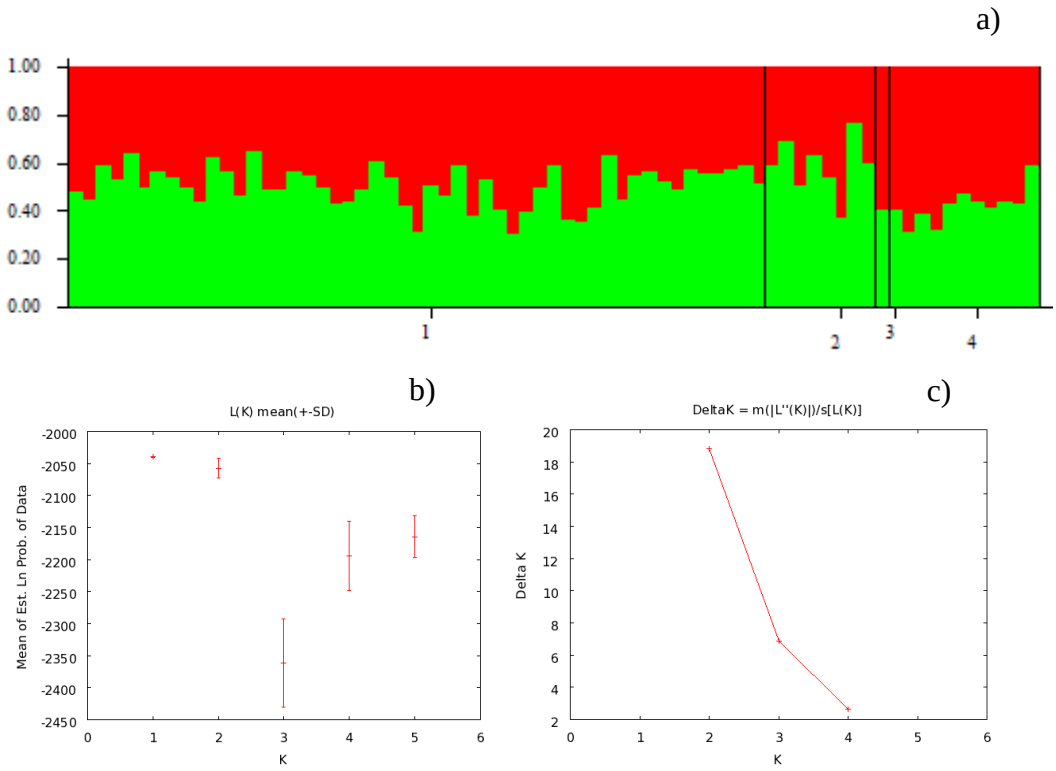


Fig. 4.11: result of STRUCTURE clustering at SS populations: 1= Viareggio; 2= Cagliari; 3= Alexandria (Al 21); 4) Turkish coasts.
a) bar plot with K=2; b) log-Likelihood values fro different K; c) ΔK

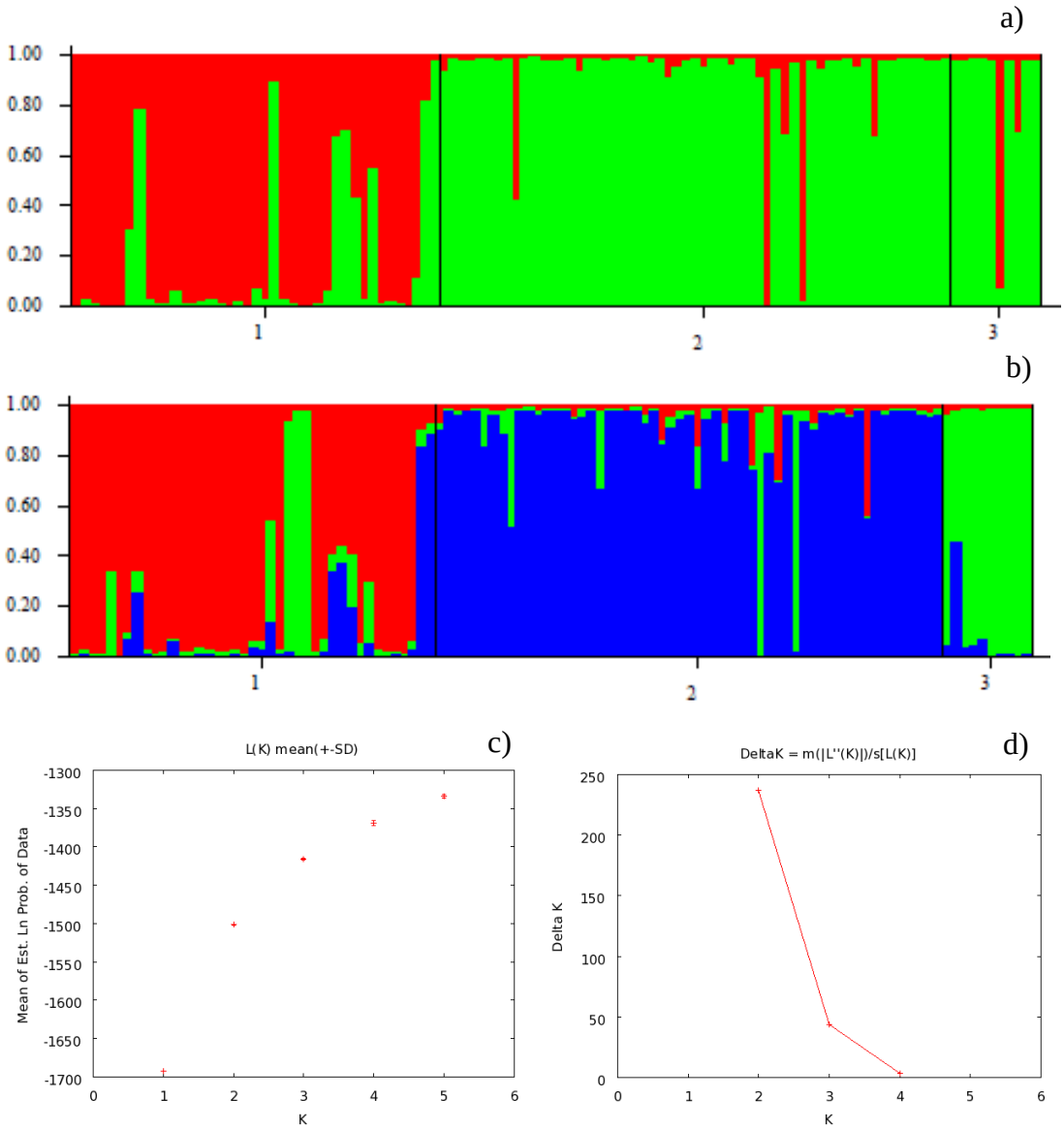


Fig. 4.12: Results of STRUCTURE clustering within SA populations: 1= Cagliari; 2= Alexandria; 3= Turkish coasts.
a) K=2; b) K=3; c) log-Likelihood values for different K and d) ΔK

In *S. solea* the clustering analysis did not separate individuals in populations (fig. 4.11), confirming the PCA results (fig. 4.10a). The bar plot (Fig. 4.11a) showed that each cluster equally contributes to the genetic composition of individuals. A weak differentiation of the Turkish coasts sample (group 4 in the x-axis) was apparent. The individual bar plot of unique *S. solea* individual AL21 from Alexandria (group 3 in the x-axis) did not differ form the others.

On the contrary, the clustering analyses of *S. aegyptiaca* individuals resolved a population structure but depending on the number of K. With K=2, the individuals are clearly separated in two clusters, corresponding to the individuals from Western Mediterranean population sample of Lagoons of Cagliari (the group 1 of Fig. 4.12a) and

those from the Eastern Mediterranean population samples of Alexandria and Turkish coasts (the groups 2 and 3 of Fig. 4.12a). The bar plot obtained with K=3 (Fig. 4.12b) separated the *S. aegyptiaca* individuals in three quite-well distinct clusters corresponding to the three population samples: Lagoons of Cagliari (group 1), Alexandria (group 2) and Turkish coasts (group 3).

The pairwise F_{st} values (Tab 4.4), estimated by ARLEQUIN 3.11, were significant in comparisons between samples from the Western and Eastern Mediterranean in both species. On the contrary, while Fst value between S. solea samples from the Western Mediterranean was not significant, the Fst value between Alexandria and Turkish coasts did. These results confirmed a more pronounced genetic differentiation among the samples of *S. aegyptiaca* than among those of *S. solea* samples.

Tab. 4.3 Pairwise F_{st} values between population samples of *S. solea* (SS) and *S. aegyptiaca* (SA).

SS	Viareggio	Lagoons of Cagliari
Viareggio		
Lagoons of Cagliari	0.01597	
Turkish coasts	0.05319*	0.11873*

SA	Lagoons of Cagliari	Alexandria
Lagoons of Cagliari		
Alexandria	0.10354*	
Turkish coasts	0.22500*	0.13860*

Significance level:*p<0.01

5. DISCUSSION

This study provides important advances in the molecular ecology and evolution of two closely-related sole species *S. solea* and *S. aegyptiaca*, which *i)* are important fishery resources in the Mediterranean, *ii)* exhibit high level of rough external morphology (i.e. cryptic species) and *iii)* co-occur sympatrically in several areas of the Western and Eastern Mediterranean.

Using available and newly developed molecular markers (i.e. a panel of microsatellite loci and the ITS1 locus, respectively), this thesis work has improved species identification by developing rapid and discriminating PCR-based tests and the understanding of ecological and evolutionary relationships between these two species. The technological and scientific advances can be used for improving the sustainable exploitation of these two fishery resources in the Mediterranean.

Species distribution and sympatric demes of Solea in the Mediterranean

Several studies have documented the sympatric presence of *S. solea* and *S. aegyptiaca* in some areas of the Mediterranean Sea, as the Gulf of Lions (Borsa and Quignard, 2001), the Tunisian coast and the area of Suez Channel; (She et al., 1987a; She et al, 1987b; www.fishbase.org), using cytochrome b sequence, allozyme and EPIC markers. By analysing cytb sequence variation in several Mediterranean population samples, Micheli (2011) has recently confirmed the occurrence of a sympatric deme in the Gulf of Lions but also discovered new sympatric demes in the Levantine basin (Turkish coasts), Aegean Sea (Kavala Gulf), Ionian Sea (Gulf of Taranto), North-Adriatic Sea (Slovenian coasts) and Tyrrhenian Sea (Lagoons of Cagliari). The genetic survey I have carried out based on nuclear markers confirmed the occurrence of the Egyptian sole in high frequency in the mixed demes of the Levantine and Tyrrhenian Seas (Turkish coasts and Lagoons of Cagliari, respectively). In addition, my work has newly shown that in Alexandria (the typical known area of occurrence of the Egyptian sole) also *S. solea* might rarely occur. The assignment of the individual AL21 to the species *S. solea* is supported by all three markers (ITS1, microsatellites and cytb haplotype). The multilocus microsatellite genotype of this individual is closely related to those of the common soles from Turkish coasts and therefore it can be argued that the soles inhabiting the Mediterranean coasts of Turkey can disperse southward along the shelves of Lebanon, Israel and Egypt. It is quite interesting also that some demes are not mixed (e.g. Viareggio, North Tyrrhenian). Nevertheless it can not be ruled out that this area is

inhabited only by *S. solea*, as the documented occurrence of *S. aegyptiaca* in relatively close sites as the Orbetello Lagoons (Micheli 2011;), the Lagoons of Cagliari and the Gulf of Lions might suggest that *S. aegyptiaca* was not sampled in the North Tyrrhenian Sea but it can occur in this ecologically suitable habitat.

Further multidisciplinary data (i.e. combining data from morphology, reproductive biology and life history with genetic data of the same individual) obtained from new and more samples are needed to unravel distribution and ecology of *S. aegyptiaca* in the Mediterranean and its ecological interactions with the cryptic species *S. solea*. However, some alternative hypothesis can be addressed to interpret this scenario. The first hypothesis is that the distribution and ecology of *S. aegyptiaca* isn't yet completely unravelled: since now this species could have been frequently misidentified with the cryptic *S. solea* and their life history traits were not properly documented. Only in the recent years the development and application of molecular tools enabled the identification of *S. aegyptiaca* (She et al., 1987a; She et al., 1987b; Borsa and Quignard, 2001; Vachon et al., 2008) and therefore any future bio-ecological study and fishery abundance estimates on these species should require an initial molecular-based species identification (see the paragraph *Marker efficiency in the identification of sole taxa*).

The second fascinating hypothesis is that historical environmental changes could have played a significant role in modifying the Egyptian sole distribution in the Mediterranean. The change of environmental conditions, such as the increasing of water temperature in the Mediterranean (Bianchi and Morri, 2003), along with other anthropogenic factors, as trawl fishery and aquaculture, causing alteration of habitats, translocation of organisms and species movement (Rhymer and Simberloff 1996; Johnson 2000; Simons *et al*. 2001; Allendorf *et al.* 2001) might have driven an expansion of the distribution area of *S. aegyptiaca* westward and northward in the Mediterranean from a dispersal centre located in the South-eastern corner. In the marine ecosystems, warmer temperatures are causing rapid northwards expansion of species adapted to temperate climate (Machado-Schiaffino et al., 2010).

Reproductive isolation of S. solea and S. aegyptiaca

The wide sympatric occurrence of the two closely related sole species in the Mediterranean led to challenging evolutionary questions whose resolution might have relevant downstream consequences on the conservation and sustainable fishery of the two species.

Secondary sympatry of species allows possible interspecific reproductive interactions and, whether pre-zygotic and post-zygotic reproductive barriers were overwhelmed, interspecific hybrid F_1 progeny can be produced. In addition, whether this hybrid progeny is fertile and back-crosses with parental species, maternal or paternal genes/genomes of the hybrids can be introgressed in the gene pool of the parental species. In the case that the gene/genome introgressed would be heterospecific with respect to that of the parental species, it can be easily detected by using DNA nuclear codominant markers. In marine bony fish and even in flatfish, hybridization and gene introgression events have occurred and they were detected thanks to the use of nuclear codominant molecular markers paired to the analysis of life history traits. In flatfish, beside the detection of hybrids between *S. senegalensis* and *S. aegyptiaca* documented by She et al. (1987b) and Ouanes et al. (2011), F_1 hybrids were also found in plaices with parental reciprocal crosses (Kiewska et al 2009). In tunas, past hybridization events with the production of fertile F_1 progenies occurred between the albacore *Thunnus alalunga* and the Atlantic bluefin tuna *T. thynnus* supported by the finding of the introgression of the albacore mtDNA in the bluefin individuals and populations (Alvarado Bremer et al. 2005; Boustany et al 2008; Ferrara et al, in preparation). Other catchy cases of successful interspecific reproductive interactions in marine fish were documented, for example, in the European sturgeons (Ludwig et al. 2003) and the North American hakes (Machado-Schiaffino et al. 2010).

The results here obtained using all the analytical approaches on the microsatellite datasets consistently revealed a clear genetic separation of the two species. The multilocus F_{st} estimate and the almost all single-locus F_{st} values were high and significant indicating a complete lacking of gene flow among taxa. Individual multilocus genotypes were grouped by PCA and clustered by the Bayesian model based method in two well-distinct groups corresponding to the putative species. Again, the 54 sole individuals whose putative species was not validated by cytb haplotypes were assigned to the two species accordingly to the multiplex PCR ITS1 species identification. The multilocus assignment tests always addressed all the 179 specimens to the two putative species with very high percentages of correct assignment.

No intermediate multilocus genotypes, (i.e. admixed genotypes with equal genetic contribution from both *S. solea* and *S. aegyptiaca* clusters) that could identify putative F_1 hybrids, were found in any of the four demes. This result, coupled to the consistency between species identification obtained with nuclear codominant markers and that

obtained with cytb haplotypes by Micheli (2011), ruled out definitely past and actual hybridization events, reinforcing the issue of reproductive isolation between the Mediterranean populations of *S. solea* and *S. aegyptiaca* inferred by Borsa e Quignard (2001) and She et al. (1987). A few factors can be invoked to support such important evolutionary issue. Although the two species are closely related and share high levels of morphological similarity (i.e. cryptic species), the great genetic divergence at the loci investigated suggested a high divergence of the species-specific genomes. Post-zygotic barriers caused by genome impairing in the zygote-embryonic developmental might allowed to not-viable (and therefore non-detectable) F_1 hybrid progeny stages (Machado-Schiaffino et al., 2010). The finding of relatively high rates of hybridization between *S. aegyptiaca* and *S. senegalensis* (She et al., 1987a; Borsa e Quignard 2001; Ouanes et al., 2011), which are less genetically divergent (they are sister species) might speak in favour of an important role of genomic divergence in the production of F_1 viable progeny. However, the onset of pre-zygotic barriers which prevent the cross-specific mating might be possible. Pre-zygotic barriers can be driven by ecological and behavioural forces and factors, such as different spawning areas and seasons, different mating-recognition systems and signals (Bickford et al., 2006). The need to increase the knowledge on life history traits of *S. aegyptiaca* and to improve those of the congeneric cryptic *S. solea* throughout more extensive and deeper analyses is again a priority for better understanding the nature, the role and the efficiency of the reproductive barriers in maintaining genetically isolated the two sole species.

As a corollary, the results of the multiplex PCR ITS1 assay might also be of interest for understanding the molecular evolution of the rDNA multicopy genes in the *Solea* species. The co-amplification of a *S. solea* specific 193-bp band together with the 602-bp band specific of *S. aegyptiaca* in the multiplexed PCR ITS1 profile of all *S. aegyptiaca* individuals (whose species identification was validated by both microsatellite and cytb markers) suggests that some *S. solea* –like rDNA gene clusters might be present in the nuclear genomes of the more recently-derived species *S. aegyptiaca*. Although the *S. solea* species-specific primer pair ITS1F-SSr3 didn't give any cross-amplification in *S. aegyptiaca* in the *in silico* PCR tests, it can not be excluded that an incomplete sequence homogenization (Charlesworth et al. 1994) of rDNA multigene family has been occurred in *S. aegyptiaca*.

A case study on the functional human centromeres (Schueler et al., 2001) documented the presence in the pericentromeric area (which is rich of repetitive highly-repetitive

DNA satellite sequences) of an ancestral primate specific sequence of α-satellite DNA family. In this case, more ancient sequences of this family derived from the primate ancestor coexist with de-novo sequences attendant only in the derived species and not found in the ancestor. In soles, a parallel evolutionary scenario could have occurred for the rDNA multigene family of *Solea*, being *S. aegyptiaca* the derived taxon that still possesses rDNA sequences of the ancestor taxon *S. solea*. At any rate, additional lab tests such as cloning and sequencing of the amplified PCR fragments in both species are required to confirm this hypothesis. In addition, the same lab work should be carried out on the other closely related congeneric species as *S. senegalensis*.

Marker efficiency in the identification of sole taxa

The development and use of high-efficiency markers have considerable downstream relevance for improving sustainable fishery. For example, the use of genetic markers highly performing at the between-population level might allows the identification of distinct fishery/biological units (stock units) improving the quality and reliability of stock assessments. Again, the availability of rapid, costless and efficient markers at the species level offers the possibility to identify frauds and illegal practices in the seafood trade.

Here, I have extensively used nuclear DNA codominant markers with the aim to genetically identify sole taxa (i.e. species, populations and individuals). The panel of 8 microsatellite loci which can cross-amplify in multiple *Solea* species can be considered one of the most versatile and powerful set of molecular markers for resolving ecological and evolutionary questions at multiple taxonomic levels in this group of fish species (Rico et al. 1996, Maes et al. 2006).

In *Solea*, the panel of microsatellite markers I have used is powerful at the species level because they discriminate sole species with high level of confidence. The high percentages of correctly assigned individuals (over the 85%) obtained with the GENECLASS multilocus test at the species level highlighted the robustness of these markers in the taxon traceability. Moreover, the same results in terms of differentiation and efficiency can be obtained using few marker loci which can be considered straightforward molecular tools for resolving fishery-related questions of Mediterranean soles (i.e. the identification of cryptic species). The same panel of microsatellite loci is suitable to assign individuals to Mediterranean geographical populations in both species

and to achieve the structure of populations, even if more robustly in *S. aegyptiaca* than in *S. solea*.

Even though the multiplex PCR ITS1 assay didn't perform completely in the identification of hybrids between *S. solea* and *S. aegyptiaca* or viceversa, it has been proven as a rapid, costless and valuable tool for the *Solea* species identification, being its results 100% consistent with the microsatellite-based species assignment. The multiplexed PCR test enables the species identification of unknown specimens in one PCR step, reducing the time and the cost needed for performing molecular identification with cytb sequencing and the genotyping of microsatellite loci on a fluorescent-based analytical system.

APPENDIX 1: comparison between the results of the three molecular markers in the 179 individuals.

VIAREGGIO	**MARKER**		
specimen name	mtDNA	ITS1	STR
VG 1		solea	solea
VG 2	solea	solea	solea
VG 3	solea	solea	solea
VG 4	solea	solea	solea
VG 5	solea	solea	solea
VG 6		solea	solea
VG 7	solea	solea	solea
VG 8		solea	solea
VG 9		solea	solea
VG 10		solea	solea
VG 11		solea	solea
VG 12	solea	solea	solea
VG 13	solea	solea	solea
VG 14	solea	solea	solea
VG 15	solea	solea	solea
VG 16	solea	solea	solea
VG 17	solea	solea	solea
VG 18	solea	solea	solea
VG 19	solea	solea	solea
VG 20	solea	solea	solea
VG 21	solea	solea	solea
VG 22	solea	solea	solea
VG 23	solea	solea	solea
VG 24	solea	solea	solea
VG 25		solea	solea
VG 26	solea	solea	solea
VG 27	solea	solea	solea
VG 28	solea	solea	solea
VG 29	solea	solea	solea
VG 30	solea	solea	solea
VG 31	solea	solea	solea
VG 32		solea	solea
VG 33	solea	solea	solea

VG 34	solea	solea	solea
VG 35	solea	solea	solea
VG 36	solea	solea	solea
VG 37		solea	solea
VG 38		solea	solea
VG 39	solea	solea	solea
VG 40	solea	solea	solea
VG 41	solea	solea	solea
VG 42	solea	solea	solea
VG 43		solea	solea
VG 44	solea	solea	solea
VG 45		solea	solea
VG 46	solea	solea	solea
VG 47	solea	solea	solea
VG 48		solea	solea
VG 49		solea	solea
VG 50		solea	solea
VG 51	solea	solea	solea

LAGOONS OF CAGLIARI	**MARKER**		
specimen name	mtDNA	ITS1	STR
CA 1		aegyptiaca	aegyptiaca
CA 2	aegyptiaca	aegyptiaca	aegyptiaca
CA 3		aegyptiaca	aegyptiaca
CA 4	aegyptiaca	aegyptiaca	aegyptiaca
CA 5	aegyptiaca	aegyptiaca	aegyptiaca
CA 6	aegyptiaca	aegyptiaca	aegyptiaca
CA 7	aegyptiaca	aegyptiaca	aegyptiaca
CA 8	aegyptiaca	aegyptiaca	aegyptiaca
CA 9	aegyptiaca	aegyptiaca	aegyptiaca
CA 10		aegyptiaca	aegyptiaca
CA 11		aegyptiaca	aegyptiaca
CA 12	aegyptiaca	aegyptiaca	aegyptiaca
CA 13		aegyptiaca	aegyptiaca
CA 14		aegyptiaca	aegyptiaca
CA 15		aegyptiaca	aegyptiaca
CA 16		aegyptiaca	aegyptiaca
CA 17		aegyptiaca	aegyptiaca

CA 18		aegyptiaca	aegyptiaca
CA 19	solea	solea	solea
CA 20		aegyptiaca	aegyptiaca
CA 21		aegyptiaca	aegyptiaca
CA 22		aegyptiaca	aegyptiaca
CA 23	aegyptiaca	aegyptiaca	aegyptiaca
CA 24		aegyptiaca	aegyptiaca
CA 25	aegyptiaca	aegyptiaca	aegyptiaca
CA 26	aegyptiaca	aegyptiaca	aegyptiaca
CA 27		aegyptiaca	aegyptiaca
CA 28		aegyptiaca	aegyptiaca
CA 29	solea	solea	solea
CA 30		aegyptiaca	aegyptiaca
CA 31	solea	solea	solea
CA 32	solea	solea	solea
CA 33	aegyptiaca	aegyptiaca	aegyptiaca
CA 34		aegyptiaca	aegyptiaca
CA 35	aegyptiaca	aegyptiaca	aegyptiaca
CA 36		aegyptiaca	aegyptiaca
CA 37		aegyptiaca	aegyptiaca
CA 38	aegyptiaca	aegyptiaca	aegyptiaca
CA 39		solea	solea
CA 40		solea	solea
CA 41		aegyptiaca	aegyptiaca
CA 42	aegyptiaca	aegyptiaca	aegyptiaca
CA 43	solea	solea	solea
CA 44	aegyptiaca	aegyptiaca	aegyptiaca
CA 45		aegyptiaca	aegyptiaca
CA 46	aegyptiaca	aegyptiaca	aegyptiaca
CA 47	solea	solea	solea
CA 48	aegyptiaca	aegyptiaca	aegyptiaca
CA 49		aegyptiaca	aegyptiaca

ALEXANDRIA	**MARKER**		
specimen name	mtDNA	ITS1	STR
AL 1	aegyptiaca	aegyptiaca	aegyptiaca
AL 2	aegyptiaca	aegyptiaca	aegyptiaca
AL 3	aegyptiaca	aegyptiaca	aegyptiaca
AL 4	aegyptiaca	aegyptiaca	aegyptiaca

AL 5	aegyptiaca	aegyptiaca	aegyptiaca
AL 6	aegyptiaca	aegyptiaca	aegyptiaca
AL 7	aegyptiaca	aegyptiaca	aegyptiaca
AL 8	aegyptiaca	aegyptiaca	aegyptiaca
AL 9		aegyptiaca	aegyptiaca
AL 10	aegyptiaca	aegyptiaca	aegyptiaca
AL 11	aegyptiaca	aegyptiaca	aegyptiaca
AL 12	aegyptiaca	aegyptiaca	aegyptiaca
AL 13	aegyptiaca	aegyptiaca	aegyptiaca
AL 14	aegyptiaca	aegyptiaca	aegyptiaca
AL 15	aegyptiaca	aegyptiaca	aegyptiaca
AL 16	aegyptiaca	aegyptiaca	aegyptiaca
AL 17	aegyptiaca	aegyptiaca	aegyptiaca
AL 18	aegyptiaca	aegyptiaca	aegyptiaca
AL 19	aegyptiaca	aegyptiaca	aegyptiaca
AL 20	aegyptiaca	aegyptiaca	aegyptiaca
AL 21	solea	solea	solea
AL 22	aegyptiaca	aegyptiaca	aegyptiaca
AL 23	aegyptiaca	aegyptiaca	aegyptiaca
AL 24	aegyptiaca	aegyptiaca	aegyptiaca
AL 25		aegyptiaca	aegyptiaca
AL 26	aegyptiaca	aegyptiaca	aegyptiaca
AL 27	aegyptiaca	aegyptiaca	aegyptiaca
AL 28	aegyptiaca	aegyptiaca	aegyptiaca
AL 29	aegyptiaca	aegyptiaca	aegyptiaca
AL 30	aegyptiaca	aegyptiaca	aegyptiaca
AL 31	aegyptiaca	aegyptiaca	aegyptiaca
AL 32	aegyptiaca	aegyptiaca	aegyptiaca
AL 33	aegyptiaca	aegyptiaca	aegyptiaca
AL 34	aegyptiaca	aegyptiaca	aegyptiaca
AL 35	aegyptiaca	aegyptiaca	aegyptiaca
AL 36	aegyptiaca	aegyptiaca	aegyptiaca
AL 37	aegyptiaca	aegyptiaca	aegyptiaca
AL 38		aegyptiaca	solea
AL 39	aegyptiaca	aegyptiaca	aegyptiaca
AL 40		aegyptiaca	aegyptiaca
AL 41	aegyptiaca	aegyptiaca	aegyptiaca
AL 42		aegyptiaca	aegyptiaca
AL 43		aegyptiaca	aegyptiaca

AL 44		aegyptiaca	aegyptiaca
AL 45		aegyptiaca	aegyptiaca
AL 46	aegyptiaca	aegyptiaca	aegyptiaca
AL 47		aegyptiaca	aegyptiaca
AL 48		aegyptiaca	aegyptiaca
AL 49		aegyptiaca	aegyptiaca
AL 50		aegyptiaca	aegyptiaca
AL 51	aegyptiaca	aegyptiaca	aegyptiaca
AL 52	aegyptiaca	aegyptiaca	aegyptiaca
AL 53	aegyptiaca	aegyptiaca	aegyptiaca
AL 54	aegyptiaca	aegyptiaca	aegyptiaca
AL 55	aegyptiaca	aegyptiaca	aegyptiaca
AL 56	aegyptiaca	aegyptiaca	aegyptiaca
AL 57		aegyptiaca	aegyptiaca
AL 58		aegyptiaca	aegyptiaca

TURKISH COASTS	**MARKER**		
specimen name	mtDNA	ITS1	STR
AK 1	solea	solea	solea
AK 2	aegyptiaca	aegyptiaca	aegyptiaca
AK 3	solea	solea	solea
AK 4	solea	solea	solea
AK 5	solea	solea	solea
AK 6	solea	solea	solea
AK 7	solea	solea	solea
AK 8	solea	solea	solea
AK 9	solea	solea	solea
AK 10	solea	solea	solea
AK 11	solea	solea	solea
AN 1	aegyptiaca	aegyptiaca	aegyptiaca
AN 2	aegyptiaca	aegyptiaca	aegyptiaca
AN 3	aegyptiaca	aegyptiaca	aegyptiaca
AN 4	aegyptiaca	aegyptiaca	aegyptiaca
AN 5	solea	solea	solea
AN 6	aegyptiaca	aegyptiaca	aegyptiaca
AN 7	aegyptiaca	aegyptiaca	aegyptiaca
AN 8	aegyptiaca	aegyptiaca	aegyptiaca
AN 9	aegyptiaca	aegyptiaca	aegyptiaca

AN 10	aegyptiaca	aegyptiaca	aegyptiaca

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