Univerzita Karlova
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Studijní program: Biologie
Studijní obor: Ekologie

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Porovnání genetické variability geograficky vzdálených populací vybraných evropských mořských druhů hlaváčů

Comparison of the genetic variability of geographically distant populations of selected species of European marine gobies

Diplomová práce

Školitelka: RNDr. Jasna Vukić, Ph.D.

Praha, 2018

Declaration:

I hereby declare that this diploma thesis is entirely the result of my own work and I have acknowledged all the sources of information which I have used. This thesis has not been submitted in order to obtain the same or any other academic degree.

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 13. 8. 2018

Podpis

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Last but not least, I would like to express my sincere gratitude to my family and my partner who were very supportive and emphatic and understood my busyness during this period.

Abstrakt

Hlaváči (Gobiidae, Actinopterygii) jsou malé, převážně kryptické, mořské, brakické i sladkovodní ryby. Vyskytují se hojně v příbřežních vodách, i když jejich výskyt zdaleka není zmapován díky jejich nenápadnosti, často skrytému způsobu života a komerční nevyužitelnosti. Informace o rozšíření mnoha druhů jsou proto stále značně neúplné. Řada druhů je známá pouze z několika málo lokalit, roztroušených po celém Středozemním moři a Atlantiku. To naznačuje, že většina druhů by mohla mít ve skutečnosti poměrně souvislý areál výskytu. Srovnání genetické variability geograficky vzdálených populací stejného druhu může pomoci odhalit, zda dochází k diferenciaci populací a zda jsou populace od sebe geneticky izolované. Cílem práce bylo porovnat genetickou variabilitu populací osmi druhů hlaváčů: *Corcyrogobius liechtensteini, Gobius cruentatus, Gobius geniporus, Gobius incognitus*, *Chromogobius quadrivittatus, Chromogobius zebratus, Millerigobius macrocephalus* a *Zebrus zebrus* v rámci Středozemního moře a severo-východního Atlantického oceánu. Použité vzorky v této práci byly nasbírané na dvou lokalitách v Atlantickém oceánu (Španělsko a Portugalsko) a sedmi lokalitách ve Středozemním moři (Francie, Sicílie, Chorvatsko, Černá hora, Řecko, a Kypr - severní a jižní část). Tato studie byla založena na molekulárně-genetických metodách. Bylo zanalyzováno kolem 350 jedinců pro mitochondriální marker cytochrom b (cyt b) a nukleární marker ribozomální protein gen S7. Výsledky ukazují, že u většiny druhů je cyt b polymorfnější, s výjimkou *C. liechtensteini*, u kterého byl polymorfnější marker S7. U žádného z těchto druhů nebylo zaznamenáno zřetelné rozdělení populací, spíše jistý gradient, tj. rozdíl v genetické rozmanitosti mezi (sub)populacemi z lokalit na nejvýchodnějším a nejzápadnějším konci distribuce. Pro *G. cruentatus* a *G. geniporus* byla zjištěna určitá míra genetického rozdělení populací, naznačující potenciální existenci geografických bariér. Zdá se, že Sicilský kanál, který je považován za důležitou bariéru pro tok genů u některých představitelů mořské fauny, by mohl hrát roli v utváření genetické struktury některých ze studovaných druhů. Vzhledem k tomu, že dospělí hlaváči nemigrují, je pasivní transport planktonických larev pravděpodobně velmi důležitý pro genetickou strukturu populací studovaných druhů hlaváčů. Kromě toho byla odhalena skrytá rozmanitost ve dvou liniích kryptobentických hlaváčů, kde byla zjištěna existence tří neznámých druhů.

Klíčová slova:

Actinopterygii, ryby, Gobiidae, Středozemní moře, populační genetika, evoluce

Abstract

Gobies (Gobiidae, Actinopterygii) are small, predominantly cryptic, marine, brackish and freshwater fishes. They abundantly inhabit coastal waters, although their occurrence is far from being mapped due to their inconspicity, often hidden lifestyle and a lack of commercial use. Information about distribution of many species is therefore still rather poor. Many species are known only from a few locations scattered throughout the Mediterranean Sea and the Atlantic. This suggests that most species could actually have a relatively continuous distribution area. Comparing the genetic variability of geographically distant populations of the same species can help to detect whether there is some population subdivision and whether populations are genetically isolated from each other. The aim of my thesis was to compare genetic variability of the populations of eight goby species: *Corcyrogobius liechtensteini, Gobius cruentatus, Gobius geniporus, Gobius incognitus*, *Chromogobius quadrivittatus, Chromogobius zebratus, Millerigobius macrocephalus* and *Zebrus zebrus* within Mediterranean Sea and north-eastern Atlantic Ocean. Samples used in this work were collected from two Atlantic (Spain and Portugal) and seven Mediterranean localities (France, Sicily, Croatia, Montenegro, Greece, and Cyprus - northern and southern part). This study was based on molecular-genetic methods. Around 350 specimens were analysed for both mitochondrial marker cytochrome b (cyt b) and nuclear marker ribosomal protein gene S7. The results show that there was a greater polymorphism in cyt b in almost all species, except of *C. liechtensteini* that was more polymorphic in S7. No particular population subdivision has been found in any of the species, there was rather certain gradient, i.e. a difference in genetic diversity between the (sub)populations from the localities on the easternmost and westernmost end of the distribution of most of the studied species. Certain degree of genetic partitioning and potential existence of geographic barriers was found for *G. cruentatus* and *G. geniporus*. It seems that Sicily Channel, which has been evidenced as an important breakpoint for gene flow in some representatives of marine fauna, could play a role in genetic structuring in some of the studied species. Due to the fact that adult gobies do not migrate, passive transport of planktonic larvae is probably very essential for the genetic structure of populations of studied species of gobies. Additionally, a cryptic hidden diversity was revealed in two lineages of cryptobenthic gobies where three unknown species have been detected.

Key words:

Actinopterygii, fish, Gobiidae, Mediterranean Sea, population genetics, evolution

Content

Introduction

Marine organisms can show strong genetic differentiation and population structure despite of the high connectivity and concept of open seas (Pascual et al., 2017). The key role in the formation of the structure of inter- and intraspecific differentiation is managed by various molecular, genetic, climatic, geomorphological and historical factors along with the specific characteristics of individual species. Geographical structure in different organisms on intraspecific level has confirmed an evidence of existence of phylogeographical breaks in the marine environment (Longhurst, 1998). Some of the driving forces of genetic variability are considered to be mainly oceanographic barriers, marine currents, habitat discontinuities, life-history traits, larval pelagic phases, migratory behaviour of adults, isolation by distance and limited dispersal capabilities (Charrier et al., 2006; Palumbi, 1994; Schunter et al., 2011). Final scenario of genetic partitioning can be the consequence of one or interaction of more factors. Understanding these processes is crucial for marine phylogeographical investigations.

The delimitation of individual populations in the ocean area is particularly difficult because barriers to gene flow are far less apparent in marine species than in terrestrial and freshwater species (Patarnello et al., 2007). Vicariant speciation is the most frequent type of emergence of new species indicating genetic discontinuities across geographical ranges (Cunningham and Collins, 1998). It separates populations unevenly across a given boundary because the population structure of taxa with higher dispersal activities is less noticeable. Many studies have been done recently which prove that even the ocean is fragmented habitat and many species thus exhibit a spatial genetic differentiation (Palumbi, 1994). Within the zoogeographical region of the north-eastern Atlantic and the Mediterranean, different phylogeographic structures were found for distinct species, including some closely related species (Bargelloni et al., 2005; Charrier et al., 2006; Zardoya et al., 2004). Dissimilar influence of individual current or former biogeographical barriers have also been identified for different species (Patarnello et al., 2007). The Mediterranean Sea is an ideal study area because the circulation process is well documented (Fernández et al., 2005; Rio et al., 2007).

North-eastern Atlantic, Mediterranean Sea and oceanographic barriers

The European seas belong to a single zoogeographical area. This includes the Northeast Atlantic, the Mediterranean and the Black Sea (Kovačić and Patzner, 2011). Within this region there are several oceanographic breaks known. Major front in north-eastern Atlantic is created by Azores current, originating as the eastern branch of the Gulf Stream. This current separates saltier water belonging to the Subtropical Gyre (STG) from the northern colder Eastern North Atlantic Central Water (ENACW). Azores front system is further divided into two parts, one of which is directed to the Gulf of Cadiz and second

turning south along the African coast (Alves et al., 2002; Bonfardeci et al., 2018; Rogerson, 2012) (see Fig.1).

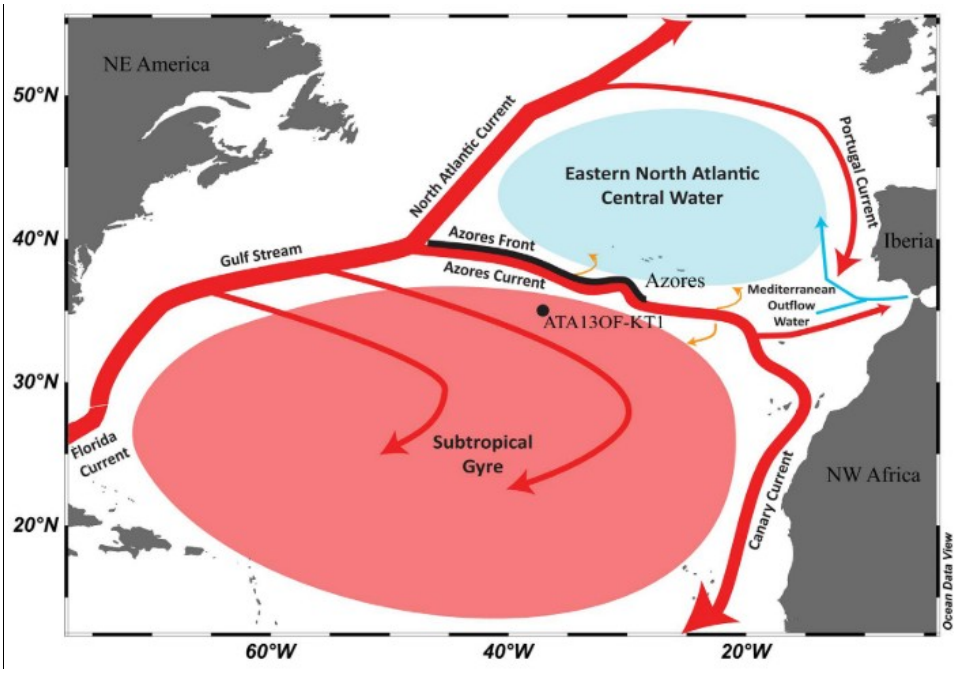

Figure 1:
Schematic surface circulation in the northern Atlantic (Bonfardeci et al., 2018).

The Mediterranean Sea represents a semi-enclosed basin with complicated circulation (Mejri et al., 2009). The gateway between the Atlantic Ocean and Mediterranean Sea is mediated by the Strait of Gibraltar (GS). This discontinuity is caused by the different salinities of these two water masses and thus can affect the connectivity between populations at both sides. The Mediterranean Sea is characterised by its high salinity compared to other seas (Emeis et al., 2000), which gradually decreases from southeast to northwest. That is given by the fact that there is an inflow of a less saline surface current of cold Atlantic oceanic water (Gysels et al., 2004a; Topper and Meijer, 2015) and hence it acts as barrier to gene flow for numerous species (Galarza et al., 2009a, 2009b). Another important break is the Almeria-Oran Front (AOF), which is considered to be the main barrier between Atlantic and Mediterranean biota. This semi-permanent front is located from Almeria (Spain) to Oran (Morocco) and is influenced by circulation in the Alboran Sea (Patarnello et al., 2007). It forms connection of two jets of Atlantic and Mediterranean water masses and creates salinity and temperature driven current flowing from the Spanish to the African coast (Tintoré et al., 1988). Another example is current flowing along the east side of Iberian peninsula and turning around the Ibiza Channel (IC) towards the Balearic Front (BF), which creates significant effect in phylogeographical patterns of fishes and crustaceans (Galarza et al., 2009b; García-Merchán et al., 2012). The western and eastern parts of the Mediterranean Sea are separated by the Sicily Channel (SC) (Lasram et al., 2009). Two peninsulas, Apennine and Balkan, divide the Mediterranean Sea into distinct marine provinces or sub-basins (Mejri et al., 2011).

Mediterranean sub-basins exhibit different hydrographic and ecological features and are variously interconnected (Agostini and Bakun, 2002). It is obvious that Sicily Channel together with Otranto Strait (OS) and Aegean Front (AEG) to some extend influence genetic structure too, however their importance was not well documented (Innocentiis et al., 2004; Serra et al., 2010; Zitari-Chatti et al., 2009) (see Fig.2).

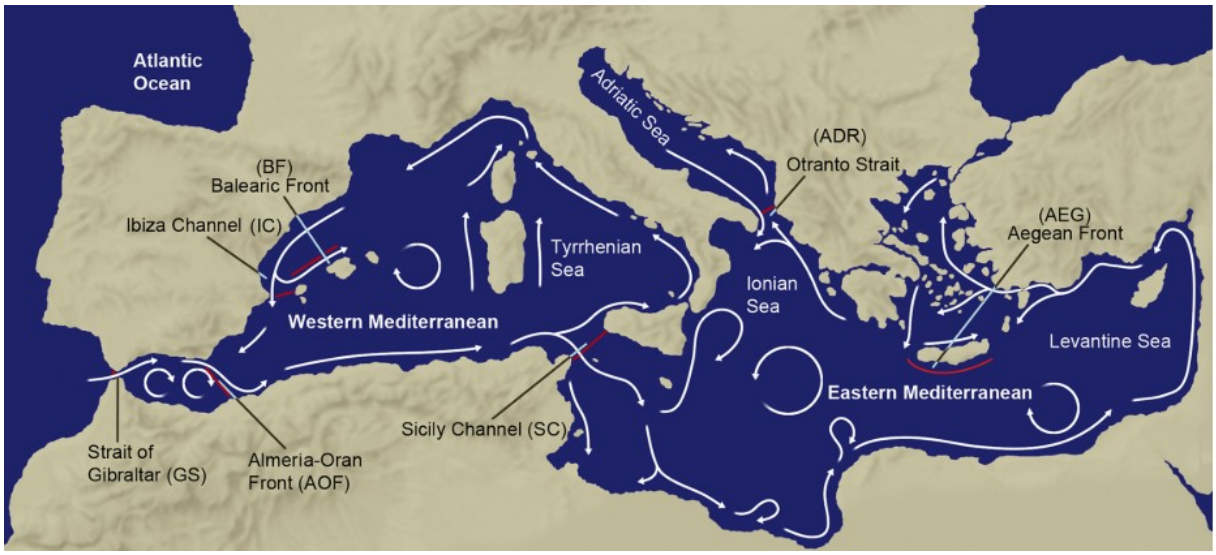

Figure 2:
Circulation of the Mediterranean Sea. White lines with arrows display main currents and red lines stand for oceanographic fronts (Pascual et al., 2017).

With the aim to understand marine connectivity in relation to larval dispersal of species caused by ocean circulation, two studies were conducted. The method used in the papers is based on connectivity approach, assessed from ensemble Lagrangian simulations of particles with oceanographic currents. This model is applied to the Mediterranean Sea with the goal to obtain information about hydrodynamical units to help to design marine protected areas network (Berline et al., 2014; Rossi et al., 2014). The disadvantage of these investigations is that they do not cover gene flow data among localities. Patarnello et al. (2007) have provided coherent view which comprises genetic data with phylogeographical patterns of Atlantic–Mediterranean transition, but does not take into account biological traits. Several studies have then been carried out, but the results or data provided do not allow a correct estimation of the impact of oceanographic fronts on the gene flow. First comprehensive study has been reported by Pascual et al. (2017). This review underlines the global effect of the oceanographic discontinuities on population differentiation but depending on life history characteristics of species. Their results show that the effect of the oceanographic fronts reduces gene flow in pelagic species with longer planktonic larval duration (2–4 weeks). On the other side, benthic species and/or with short planktonic larval durations (< 2 weeks) have more considerable genetic differentiation than species with higher motility, but independent of barriers.

Biogeographical origins of the Mediterranean biota

Biodiversity of the Mediterranean Sea is considered to be one of the richest in the world (Coll et al., 2010). In total, it contains more than 20,000 species, including plant and animal realm and more than 8,500 species of macroscopic organisms (Bianchi and Morri, 2000; Longhurst, 1998). It represents between 4% and 18% of the world's marine biodiversity with the large proportion of endemism (around 25%) (Tortonese, 1985). Therefore, the Mediterranean Sea is outlined as a biodiversity hotspot for conservation priorities. The reason of such a high biodiversity might be rooted in historical importance of Mediterranean Sea, in some former geological characteristics or also in its climatic and hydrologic variation (Bianchi and Morri, 2000).

In the late Miocene, the collision of African and Euroasian plates escalated in the desiccation of the Mediterranean known as "Messinian salinity crisis". It happened about 5.96 million to 5.33 years ago (Hsü et al., 1977) and it was accompanied by a large animal extinction (Bianchi and Morri, 2000). Evaporation caused a drop in a sea level about 1.5 kilometres (Hsü et al., 1973). As a consequence, almost all the former marine fauna disappeared. Later, in "Lago Mare" phase of "salinity crisis", the Mediterranean and Paratethys Seas transformed the structure and created large evaporitic lakes (Bianchi and Morri, 2000; Kovačić and Patzner, 2011). Since the end of the Miocene the Strait of Gibraltar created the connection between the western part of Mediterranean Sea and the Atlantic Ocean (Blanc, 2002). After the opening of the Strait of Gibraltar, a massive invasion of marine biota of Atlantic origin began to occupy the Mediterranean (Goren, 2009) what led to the evolution of Mediterranean biota and their rapid radiation (Huyse et al., 2004). This colonisation probably occurred repeatedly. However, according to the hypothesis of Garcia-Castellanos and Villaseñor (2011), the connection of the Mediterranean Sea with the Atlantic Ocean was not entirely lost during the Messinian salinity crisis. The sea level drawdown during the early stages of the crisis was only moderate (Garcia-Castellanos and Villaseñor, 2011). For example, there is a case of killifishes that have survived in shallow-water refuges (Hrbek and Meyer, 2003). New geological studies support the fact that the Mediterranean was never completely desiccated and the biota did not disappear (Garcia-Castellanos and Villaseñor, 2011; Ivanovic et al., 2014).

During Pleistocene glaciation, the alternation of the glacial and warmer interglacial phases resulted in invasion of the Mediterranean which acted like a refuge for warm water Atlantic fauna of boreal or subtropical origin (Almada et al., 2001; Bianchi and Morri, 2000). During this period of alterations of climatic conditions and sea level, the colonisation of the Mediterranean Sea probably occurred repeatedly. The next important milestone was opening of the Suez Canal in 1869, where organisms from the Red Sea started to migrate into the Mediterranean Sea known as Lessepsian immigrants (Kovačić and Patzner, 2011). On this account, the current biota is the result of a series of speciation and colonization events from different areas.

Family Gobiidae

In my master thesis I focus on the family Gobiidae (Perciformes), which in fact belongs to one of the most numerous families of fish and vertebrates. It is a remarkable group whose representatives have been able to adapt to a variety of environments, including extreme. Simultaneously, it is the most speciose group of fishes of European seas and within the Mediterranean region it comprises more than 60 species of gobies (Kovačić and Patzner, 2011; Miller, 1986). Furthermore, around other 40 species of European gobies are adapted to freshwater or brackish environments (Kottelat and Freyhof, 2007; Miller, 2003, 2004). Besides that, there are non-native species, known as lessepsian migrants (Salameh et al., 2010), which invade from the Red Sea via the Suez Canal to the Mediterranean Sea (Kovačić and Patzner, 2011). However, there are also known cases of introductions probably associated with the ballast water (Goren, 2009; Thiel et al., 2012). Even within the Mediterranean Sea, there are significant differences in the number of known species for each sub-region. Kovačić and Patzner (2011) analyzed the data on species distribution and found out that the highest number of species is known from the Adriatic Sea and the lowest from the coastal regions of Africa. In some cases, the known localities of species occurrence rather indicate which areas are better explored then real distribution of the species (Kovačić et al., 2012a).

European gobies are small, inconspicuous fishes with the maximum length of 30 cm, but most of them reaching only up to 10 cm (Kottelat and Freyhof, 2007; Kovačić and Patzner, 2011). The published data revealed that European gobies belong to three different evolutionary lineages, which are not related to each other (Agorreta et al., 2013). They can be divided into six ecological groups. Marine species occur mainly in shallow coastal waters, so they are relatively readily available for research. On the other hand, many species belong to the group of cryptobenthic fishes. These are species living with a very hidden way of life, hiding under stones, substrates, caves or crevices in the rocks, and it is very difficult to see and catch them. In addition, these cryptobenthic species are predominantly very small, growing in adulthood to a maximum of several centimetres (Miller, 1996). Perhaps due to these traits as inconspicuity, often hidden lifestyles and low commercial utility, this group is still relatively little studied in terms of biodiversity and taxonomy, evolutionary relationships, phylogeography and biogeography. As gobies are species-rich and include species differing in distribution area sizes, life-styles and ecological requirements, they are ideal model group for studies of genetic variability and phylogeography.

Analysis of population genetic variability has been done so far in only a few species of gobies from the north-eastern Atlantic and the Mediterranean. Nevertheless, on the basis of limited information it is clear that for some studied species genetic variability among populations from different areas of occurrence is evident. Comparing the genetic variability of geographically distant populations of the same species can help to detect whether the populations are genetically isolated and whether there are biogeographical barriers that affect the spread possibilities and prevent inter-population contact.

Ecology and distribution of studied species

Following species were selected, all belonging to the *Gobius* lineage:

Corcyrogobius liechtensteini (Kolombatovic, 1891)

Corcyrogobius liechtensteini (see Fig. 3) is a common representative of small cryptobenthic gobiid fish with the maximum size of 3 cm. This species is typically found on the walls and ceilings of large caves, in cavities and crevices with the rocky bottoms. It can be found in depths of 0.5 – 40 m. Sexual maturity is reached within 1 year and total lifespan is around 2.5 years. Distribution of the species can be seen in Fig. 4 (Ahnelt and Dorda, 2004; Ahnelt et al., 1994, 1998; Engin et al., 2016; Herler et al., 1999; Kovačić, 2005; Kovačić et al., 2011, 2012b, 2012a; Miller, 1986; Patzner, 1999; Scsepka and Ahnelt, 1999).

Figure 3:
Photo of *Corcyrogobius liechtensteini* (author J. Vukić).

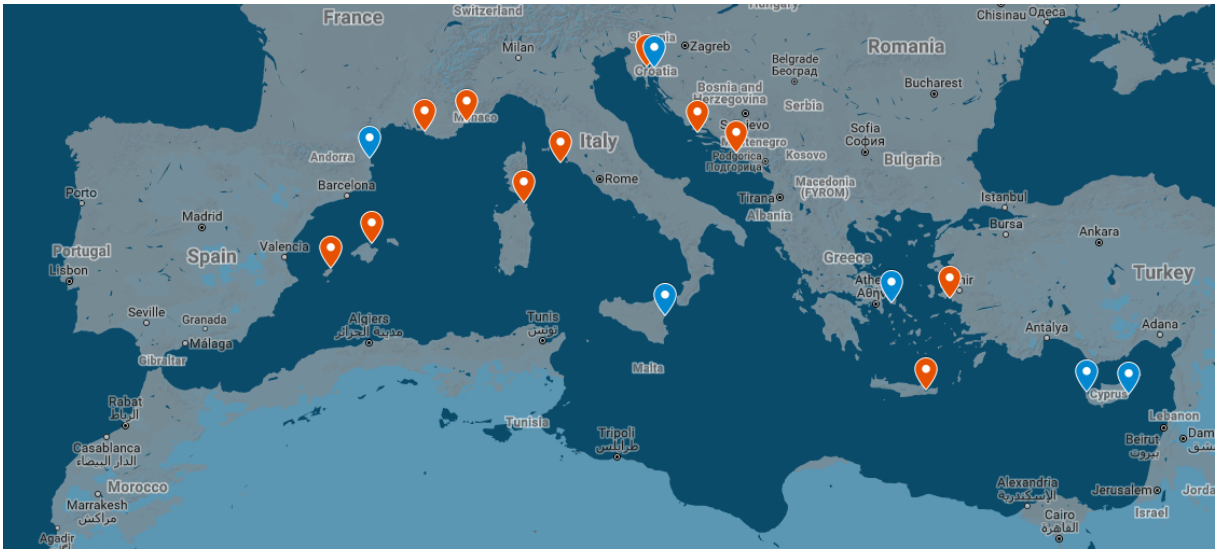

Figure 4:
Map of distribution of *Corcyrogobius liechtensteini*. Orange spots represent published known findings and blue spots respond to our collections.

Gobius cruentatus Gmelin, 1789

Gobius cruentatus (see Fig. 5) belongs to the group of epibenthic fishes, which life is closely related to the bottom. It is typically detected on mixed bottom habitats among stones, boulders or seagrass. It can grow up to 18 cm and occurs in depth from 1.5 to 40 m. Distribution of the species can be seen in Fig. 6 (Arko-Pijevac et al., 2001; Engin et al., 2007; Gil et al., 2002; Giovannotti et al., 2007; Kovačić, 2004, 2005; Miller, 1986; Sebastianutto et al., 2011; Wheeler, 1970; Wilkins and Myers, 1993).

Figure 5:
Photo of *Gobius cruentatus* (author J. Vukić).

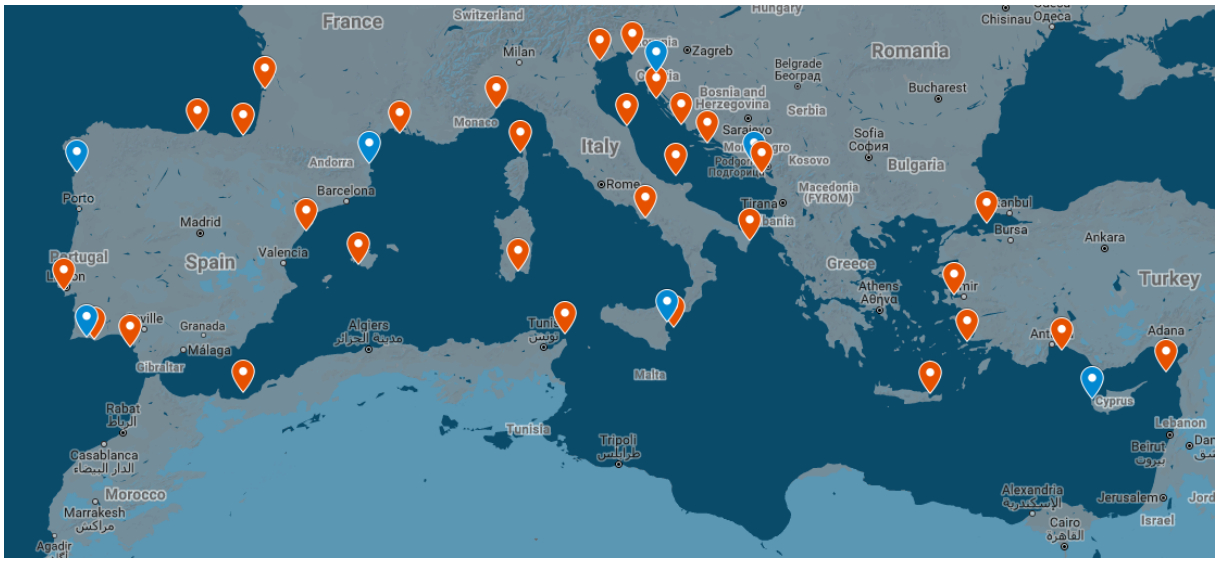

Figure 6:
Map of distribution of *Gobius cruentatus*. Orange spots represent published known findings and blue spots respond to our collections.

Gobius geniporus Valenciennes, 1837

Gobius geniporus (see Fig. 7) with the maximum body size 16 cm is relatively large bottom dwelling goby classified as epibenthic. It prefers sandy, muddy bottoms and bottoms combined of gravel, cobbles and boulders. The depth at which it was observed ranges from 1 to 30 m. Distribution of the species can be seen in Fig. 8 (Arko-Pijevac et al., 2001; Bartoli et al., 2005; Engin et al., 2016; Fricke et al., 2007; Kovačić, 2005; Kovacic and Golani; Kovačić et al., 2011; Miller, 1986).

Figure 7:
Photo of *Gobius geniporus* (author J. Vukić).

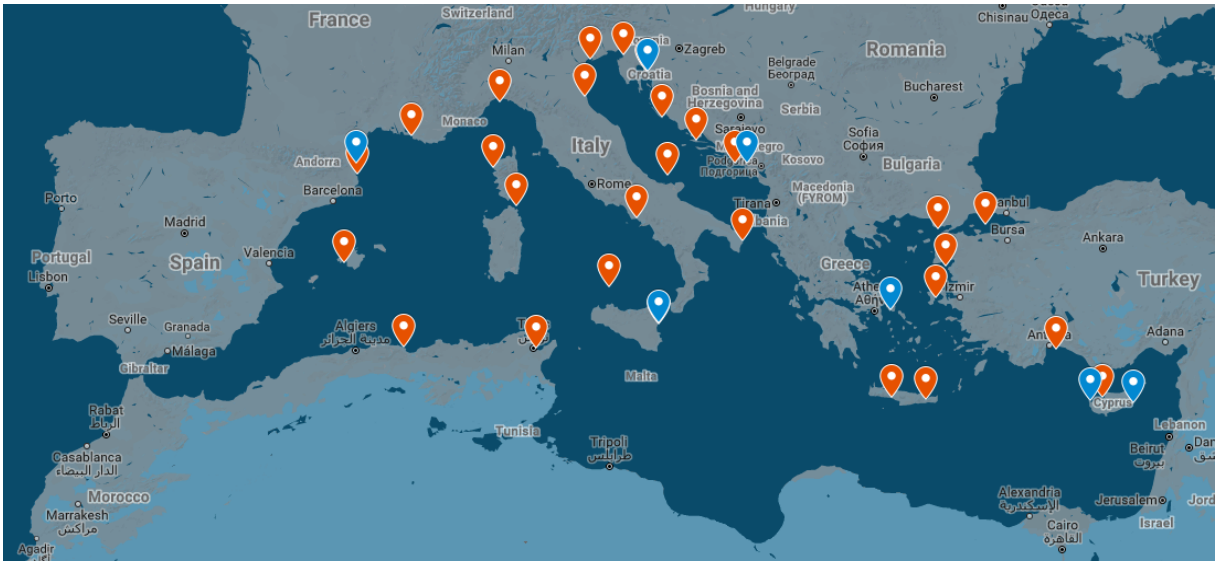

Figure 8:
Map of distribution of *Gobius geniporus*. Orange spots represent known published findings and blue spots respond to our collections.

Gobius incognitus Kovačić and Šanda, 2016

Gobius incognitus (see Fig. 9) is recently discribed epibenthic goby, which is often misidentified with morphologically and ecologically similar *G. bucchichi*. It occurs on various substrata, such as sand, gravel, cobbles, boulders and bedrock. Moreover, the substratum can be bare or covered by algae or seagrass. The depth of occurrence of this species ranges between 0.5 – 12 m and its body length is typically around 10 cm. Distribution of the species can be seen in Fig. 10 (Kovačić and Šanda, 2016).

Figure 9:
Photo of *Gobius incognitus* (author J. Vukić).

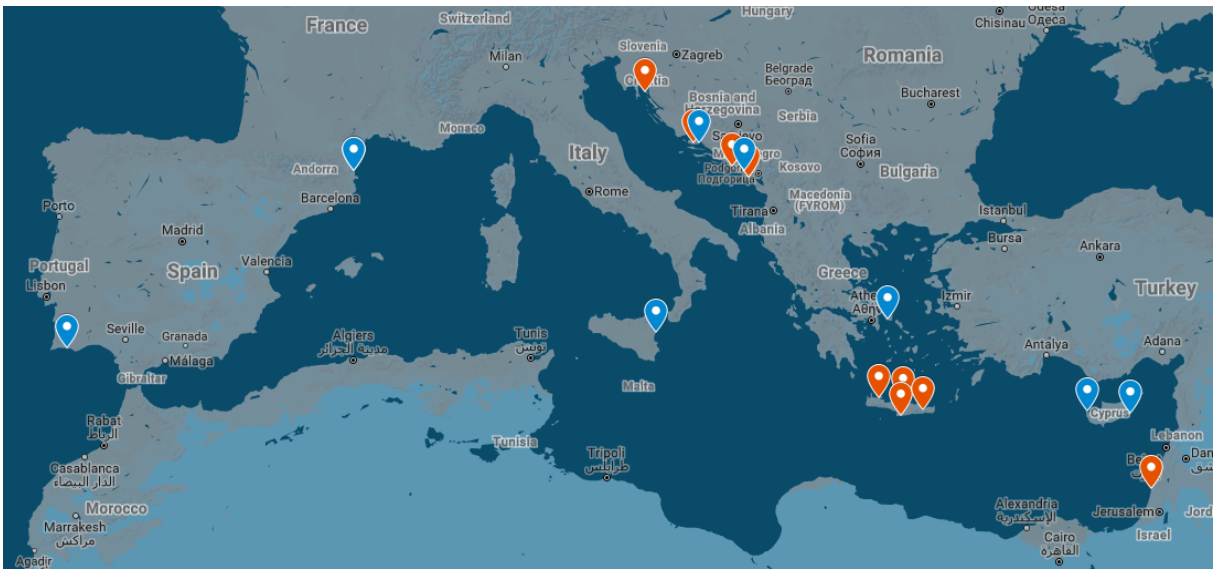

Figure 10:
Map of distribution of *Gobius incognitus*. Orange spots represent known published findings and blue spots respond to our collections.

Chromogobius quadrivittatus (Steindachner, 1863)

Chromogobius quadrivittatus (see Fig. 11) often occurs on rocky bottom with pebbles, hidden under stones and between weed tufts, it is a cryptobenthic species. The length of the body can be up to 6 cm and sexual maturity is reached in the second to third year. *Chromogobius quadrivittatus* has been seen only in a depth range of 0.3 – 3 meters. There have not been many findings of this species. Distribution of the species can be seen in Fig. 12 (Ahnelt, 1990; Colombo and Langeneck, 2013; Engin et al., 2016; Golani and Ben-Tuvia, 1986; Kovačić, 2005; Miller, 1986; Van Tassell, 2001).

Figure 11:
Photo of *Chromogobius quadrivittatus* (author J. Vukić).

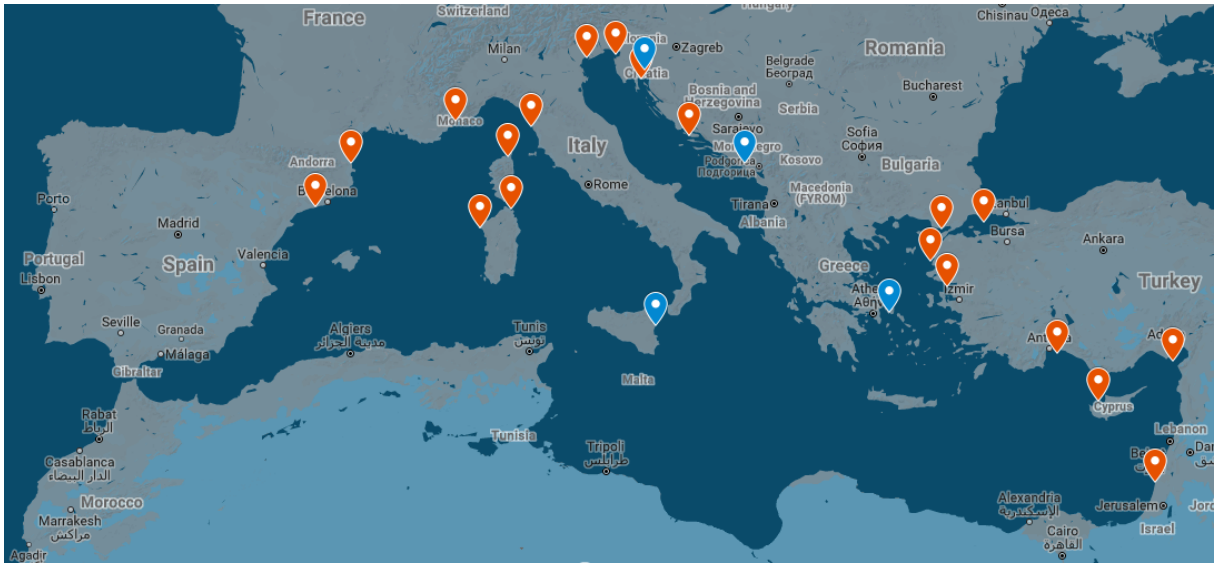

Figure 12:
Map of distribution of *Chromogobius quadrivittatus*. Orange spots represent known published findings and blue spots respond to our collections.

Chromogobius zebratus (Kolombatovic, 1891)

Chromogobius zebratus (see Fig. 13) with the body size up to 5 cm belongs to the group of cryptobenhtic fishes. It is inhabitant of boulder fields, small cavities on the bedrock and has been reported even from intertidal pools. Documented depth varies from just below the surface to up to 23 m. Distribution of the species can be seen in Fig. 14 (Ahnelt, 1990; Alberto and Nieto, 1993; Colombo and Langeneck, 2013; Engin and Dalgic, 2008; Engin et al., 2016; Kovačić, 2005; Kovačić et al., 2011, 2012b, 2012a; Miller, 1986; Patzner, 1999; Van Tassell, 2001).

Figure 13:
Photo of *Chromogobius zebratus* (author J. Vukić).

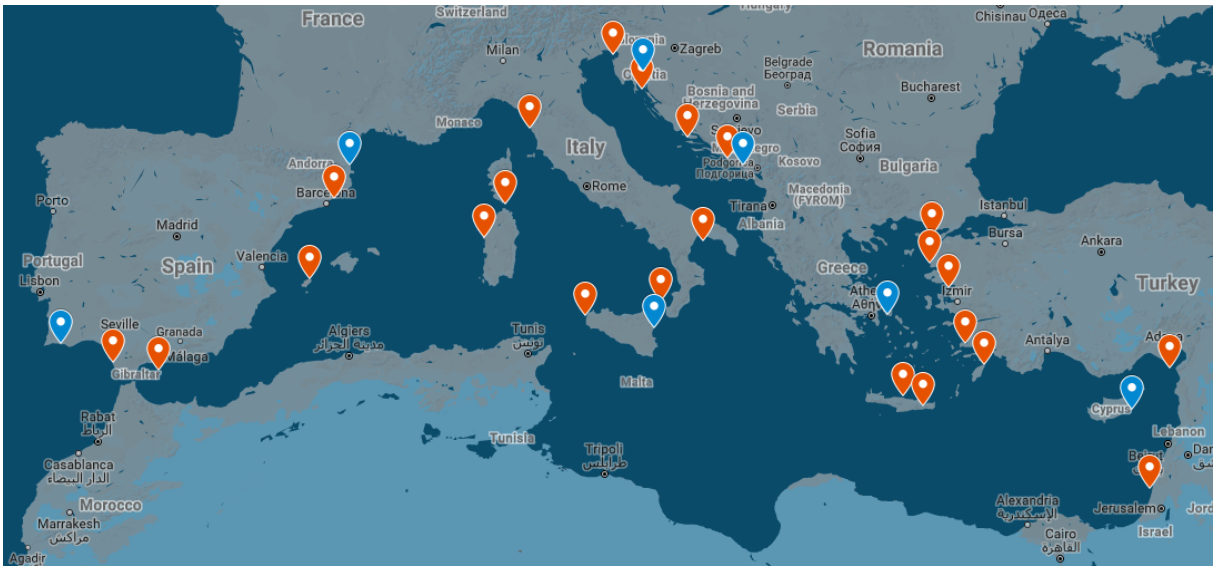

Figure 14:
Map of distribution of *Chromogobius zebratus*. Orange spots represent published known findings and blue spots respond to our collections.

Millerigobius macrocephalus (Kolombatovic, 1891)

Millerigobius macrocephalus (see Fig. 15) as well as most cryptobenhtic fishes can be found under stones, among boulders, in small cavities and caves, but also occasionally on the sandy bottoms covered with algae or under sea urchins. The total length is about 4 cm and depth of occurrence ranges from 1.5 to 25 m. Distribution of the species can be seen in Fig. 16 (Bogorodsky et al., 2010; Engin et al., 2016; Giacobbe et al., 2016; Kovačić et al., 2011, 2012b, 2012a, 2013; Miller, 1986; Patzner, 1999; Vanhove et al., 2011).

Figure 15:
Photo of *Millerigobius macrocephalus* (author F. Costa) (Patzner, 2015).

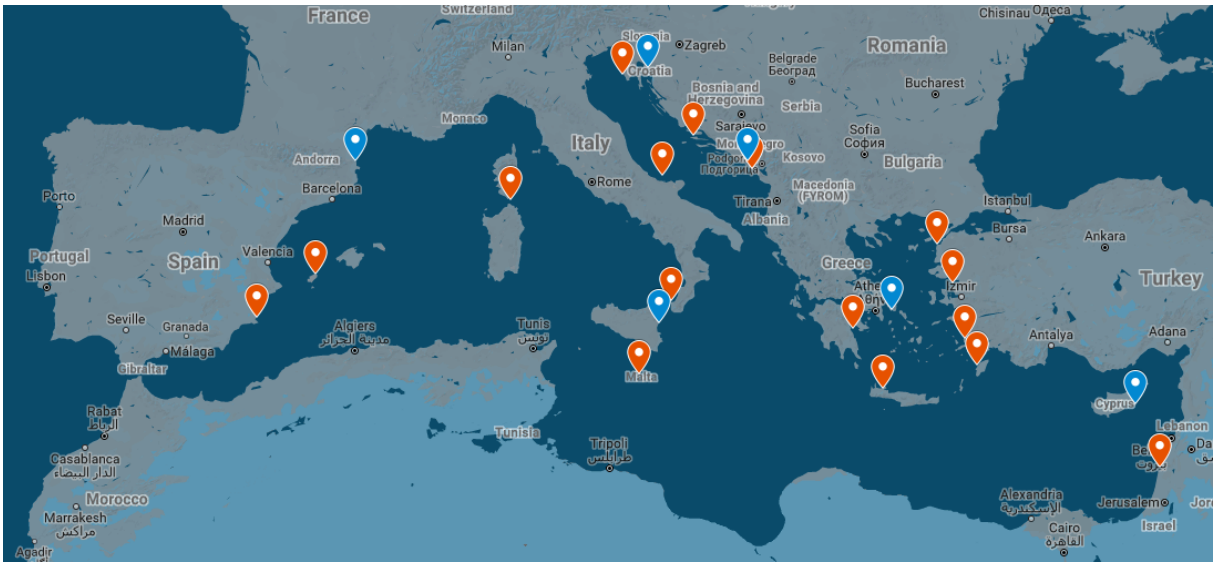

Figure 16:
Map of distribution of *Millerigobius macrocephalus*. Orange spots represent published known findings and blue spots respond to our collections.

Zebrus zebrus (Risso, 1827)

Zebrus zebrus (see Fig. 17) is also cryptobenthic. Again, it is hiding under stones, in small cavities, holes, among seagrass and furthermore there have been reports in association with sea urchin or in tidal pools. This species is documented to grow up to a size of 6 cm and live in a depth 0 – 10 m. Distribution of the species can be seen in Fig. 18 (Bogorodsky et al., 2010; Engin et al., 2016; Kovačić, 2005; Kovačić et al., 2005, 2012b, 2012a, 2013; Mejri et al., 2007; Miller, 1986; Patzner, 1999; Penzo et al., 1998).

Figure 17:
Photo of *Zebrus zebrus* (author J. Vukić).

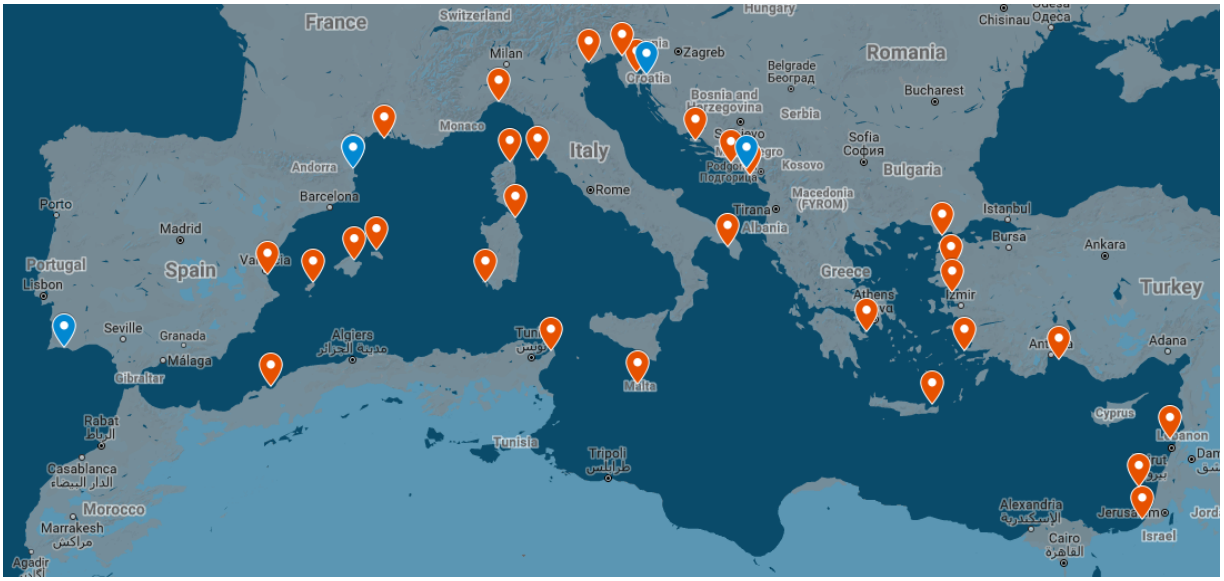

Figure 18:
Map of distribution of *Zebrus zebrus*. Orange spots represent known published findings and blue spots respond to our collections.

Aims

Main goals of my thesis are:

- to compare genetic diversity of geographically distant populations of selected species of gobies based on multilocus approach, using both mitochondrial and nuclear markers;
- to reveal a possible population subdivision;
- to reveal if genetic differences between (sup)populations indicate existence of biogeographical barrier(s) for some of the studied species.

Summary table of studied localities with corresponding species

Table 1: List of species on sampling sites with appropriate coordinates.
Spec. No. cyt b - Number of specimens for cyt b,
Hapl. No. cyt b - Number of haplotypes for cyt b
Alleles No. S7 - Number of alleles for S7
Hapl. No. S7 - Number of haplotypes for S7

Locality + coordinates	Species	Spec. No. cyt b	Hapl. No. cyt b	Alleles No. S7	Hapl. No. S7
Sicily near Catania N 37° 34' 26" E 15°10' 24"	*Corcyrogobius liechtensteini*	10	6	10	9
	Gobius cruentatus	10	9	22	2
	Gobius geniporus	11	9	28	1
	Gobius incognitus	16	13	30	8
	Chromogobius quadrivittatus	3	4		
	Chromogobius zebratus sp.1	3	3		
	Chromogobius zebratus sp.2	1	1		
	Millerigobius macrocephalus	2	1		
Greece Evia Island - southern part N 37° 59' 49" E 24° 23' 53"	*Corcyrogobius liechtensteini*	10	7	12	8
	Gobius geniporus	8	5	16	2
	Gobius incognitus	10	10	18	8
	Chromogobius quadrivittatus	2	2		
	Chromogobius zebratus sp.1	8	5		
	Chromogobius zebratus sp.2	1	1		
	Chromogobius zebratus sp.3	3	3		
	Millerigobius macrocephalus	1	1		
	Zebrus cf. *zebrus*	2	2		
Croatia Selce N 45° 9' 7" E 14° 43' 15"	*Corcyrogobius liechtensteini*	10	2	16	10
	Gobius cruentatus	11	11	22	3
	Gobius geniporus	10	9	20	1
	Chromogobius quadrivittatus	10	6		
	Chromogobius zebratus sp.1	8	8		
	Chromogobius zebratus sp.3	2	2		
Croatia Brać Island N 43° 17' 21" E 16° 52' 41"	*Gobius incognitus*	10	10	2	1
Croatia Cres Island N 44° 55' 00" E 14° 22' 55"	*Millerigobius macrocephalus*	1	1		

Montenegro Boka Kotorska N 42°29'6" E 18°40'13"	*Gobius cruentatus*	2	2	4	1
	Gobius geniporus	11	9	24	2
	Gobius incognitus	11	10	10	5
	Chromogobius quadrivittatus	15	8		
	Chromogobius zebratus sp.1	3	3		
	Millerigobius macrocephalus	4	4		
	Zebrus zebrus	4	4		
	Zebrus cf. *zebrus*	4	3		
Portugal Faro N 37° 4'26" E 8°18'13"	*Gobius cruentatus*	3	3	6	2
	Gobius incognitus	13	12	24	8
	Chromogobius zebratus sp.1	3	3		
	Zebrus zebrus	1	1		
Spain Galicia, Vigo N 42° 14'57" E 8°45'21"	*Gobius cruentatus*	5	3	4	1
France Banyuls sur Mer N 42° 28' 55" E 3° 8' 12"	*Corcyrogobius liechtensteini*	1	1	2	1
	Gobius cruentatus	3	3	6	1
	Gobius geniporus	2	2	4	1
	Gobius incognitus	12	12	18	7
	Chromogobius zebratus sp.1	4	4		
	Millerigobius macrocephalus	1	1		
	Zebrus zebrus	3	2		
Cyprus N (north) Akamas N 35° 4' 31" E 32°19' 58"	*Corcyrogobius liechtensteini*	11	5	16	10
	Gobius cruentatus	3	3	6	1
	Gobius geniporus	10	4	22	2
	Gobius incognitus	11	10	18	8
	Chromogobius zebratus sp.3	2	2		
Cyprus S (south) Cavo Greco N 34° 59' 8" E 34° 4' 36"	*Corcyrogobius liechtensteini*	6	4	12	7
	Gobius geniporus	11	9	22	2
	Gobius incognitus	10	9	12	7
	Chromogobius zebratus sp.1	3	3		
	Chromogobius zebratus sp.3	2	2		
	Millerigobius macrocephalus	4	4		

Material and methods

Sample collection

Samples were collected during years 2004 - 2017 from 8 localities within Mediterranean Sea and north-eastern Atlantic Ocean (see Fig. 19). I included in my analysis 350 specimens of 8 species, both epibenthic (*Gobius cruentatus, Gobius geniporus, Gobius incognitus*) and cryptobenthic (*Corcyrogobius liechtensteini, Chromogobius quadrivittatus, Chromogobius zebratus, Millerigobius macrocephalus, Zebrus zebrus*), at least 10 individuals of one species from ëach location was used if material was available. The sample collection was done by scuba diving with the use of anaesthetics and hand nets. A piece of tissue from the right pectoral fin was clipped and preserved in 96% ethanol for DNA analysis. Specimens were preserved in 4% formaldehyde for morphological identification and deposited in the collection of the National Museum in Prague (NMP).

Figure 19:
Map of north-eastern Atlantic and Mediterranean region with sampling sites.

Molecular methods

DNA extraction

DNA was extracted from fin tissue using *Geneaid® DNA Isolation Kit*. The Manufacturer's protocol was followed with small variations to optimize the results. The DNA isolates were then stored at -20 °C.

PCR amplification

In this study two genes were selected, one mitochondrial gene, Cytochrome b (cyt b) and one nuclear gene, intron of ribosomal protein gene S7 (S7).

Cyt b is a transmembrane protein found in mitochondria forming an important component of the respiratory chain. Its DNA sequence has a length of 1140 base pairs and it is widely used for studying taxonomy and population genetics. This mitochondrial marker is suitable for

population studies due to its sequence variability in marine fishes. This is due to fast rate of mtDNA evolution coupled with maternal inheritance. It is haploid and thus does not suffer from recombinations, introns and repetitive sequences. Cyt b exhibits intraspecific variation mostly in third position of codon which can be used for stock identifications (Habib et al., 2011; Rüber and Agorreta, 2011; Wilson et al., 1985). It has already been applied in a number of population studies in fishes (Brown and Stepien, 2008; Hsu et al., 2007).
The first intron of ribosomal protein gene S7 is a nuclear marker which is currently being used in studies of fish phylogeny and phylogeography each time more, as it shows high rate of polymorphism among the often used nuclear markers. Nuclear DNA has slower mutation rates than mtDNA. The presence of insertions and deletions makes it harder to proceed data (Annilo et al., 1995; Betancur-R et al., 2013; Zhang and Hewitt, 2003).

I amplified 350 individuals of all 8 species with mitochondrial marker cyt b (*C. liechtensteini, G. cruentatus, G. geniporus, G. incognitus*, *C. quadrivittatus, C. zebratus, M. macrocephalus, Z. zebrus*) and 223 specimens of 4 species *(Corcyrogobius liechtensteini, G. cruentatus, G. geniporus, G. incognitus*) with nuclear marker S7, in the remaining four species, this marker has not been analysed, due to lower numbers of individuals. The polymerase chain reaction (PCR) was performed in 25 µl volume using BioRad and Bioer thermocyclers. Composition of PCR master mix and reaction details are listed in Tables 2-4. Amplification of cyt b gene was done by 2 types of external primers, first by GluF and ThrR (Machordom and Doadrio, 2001), used mainly in epibenthic species, and AJG and H5 (Akihito et al., 2000), used in cryptobenthic species. However, due to occurrence of unspecific secondary products, the specific internal primers were additionally designed by our laboratory. They were used only for sequencing for all mentioned species. In case of very bad quality of PCR products or no products at all, the combination of internal and external primers was used. Nuclear gene S7 was amplified with the primers S7RPEX1F and S7RPEX2R (Chow and Hazama, 1998), referred as S7F and S7R. Sequences of all primers see in Table 5.

Table 2: Composition of PCR master mix for amplification of cyt b and S7

PCR product	**Volume** (1PCR reaction)
PPP Master Mix	12.5 µl
PCR Ultrapure H_2O	9.7 µl
Forward primer	0.65 µl
Reverse primer	0.65 µl
DNA isolate	2 µl
Final volume	**25 µl**

Table 3: PCR program for cyt b amplification

Reaction	Temperature	Time	
Initial denaturation	94 °C	3 min	
Denaturation	94 °C	45 s	} 35x
Annealing	48 °C	1 min 30 s	
Elongation	78 °C	1 min 45 s	
Final Elongation	72 °C	7 min	
Hold	4 °C	∞	

Table 4: PCR program for S7 amplification

Reaction	Temperature	Time	
Initial denaturation	95 °C	5 min	
Denaturation	94 °C	40 s	} 5x
Annealing	60 °C	1 min	
Elongation	72 °C	2 min	
Denaturation	95 °C	30 s	} 35x
Annealing	56 °C	1 min	
Elongation	72 °C	2 min	
Final Elongation	72 °C	20 min	
Hold	4 °C	∞	

Table 5: Sequences of primers used for PCR and sequencing

Primer	Direction	Sequence 5' → 3'	Reaction
GluF	forward	AAC CAC CGT TGT ATT CAA CTA CAA	PCR, sequencing
ThrR	reverse	ACC TCC GAT CTT CGG ATT ACA AGA CCG	PCR, sequencing
AJG	forward	CAA AAA CCA TCG TTG TAA TTC AACT	PCR, sequencing
H5	reverse	GAA TTY TRG CTT TGG GAG	PCR, sequencing
S7F	forward	TGG CCT CTT CCT TGG CCG TC	PCR, sequencing
S7R	reverse	AC TCG TCT GGC TTT TCG CC	PCR, sequencing
CorF1	forward	GGA TCT GAG GGG GCT TC TC	sequencing
CorR1	reverse	GAA GCM AGY AGG GCC AGT AC	sequencing
GcruF1	forward	GGT GCA ACC GTC ATC ACT AA	sequencing
GcruR1	reverse	AGT GGG TTG GCA GGA ATG	sequencing
GgenF1	forward	GTA GGC TAT GTC CTG CCC TG AG	sequencing
GgenR1	reverse	TTG GAG CCT GTC TCG TG GA	sequencing
GbucF1	forward	GGC TTC TCC GTT GAC AAT GC	sequencing
GbucR1	reverse	TGG GGG AAA AGA GGG CAA GG	sequencing
ChquaF1	forward	GTG CAA CGG TCA TCA CGA ACC	sequencing
ChquaR1	reverse	GGC CGA TGA AGT CCT TGT ACG	sequencing
ChzebF1	forward	GTC ATT CTR GCT GCC ACC CT TC	sequencing
ChzebR1	reverse	ATG TGA GGG GGG GTC ACT AG	sequencing
ZzebF1	forward	GYG CCA CMG TYA TTA CTA ACC	sequencing
ZZebR1	reverse	GAA KGG KAC TTT GTC GGA GT CG	sequencing

Electrophoresis

The size and quality of the amplified DNA products was checked by gel electrophoresis set to 150 V for 30 minutes. Gel was prepared using 1.5 % agarose, TBE buffer and fluorescent staining dye GelRed for the visualization. Subsequently, 2 µl of PCR product were applied to the gel with the reference ladder (GeneRuler 100bp Plus DNA Ladder) to determine size of the fragments.

Purification

Purification of the PCR products was done by two different methods. One was carried out by conventional ethanol precipitation method and second with the use of ExoSAP-IT.
In ethanol precipitation method, firstly, 77 µl of ultra-clean water was added to have the volume of 100 µl. Then 10 µl of sodium acetate and 200 µl of 99% ethanol (previously stored in the freezer) were added, shaked vigorously by vortexing, and centrifuged for 10 min at 14 000 rpm at room temperature. After centrifugation, supernatant was removed, 100 µl of 75% ethanol (previously stored in the freezer) was added and again mixed by vortexing. Subsequently, it was centrifuged for 10 min at 14 000 rpm and supernatant was carefully removed. The content of the tube was dried out for 1 hour at 40 °C using Heat Block, and 12 to 25 µl of ultra-clean water was added, depending on the intensity of bands on a gel. Eventually, the concentration of purified DNA was confirmed by Nanodrop spectrophotometer.

ExoSAP-IT (Exonuclease and Shrimp Alkaline Phosphatase) is relatively new cleanup reagent for amplified PCR product,which removes excess primers and unincorporated nucleotides enzymatically. Manufacturer's instructions were slightly modified in order to reduce used volume and reduce the cost. ExoSAP was diluted 10 times and 2 µl of diluted product were added to 5 µl of the PCR product and mixed. The subsequent incubation was done in thermocycler for 1 hour at 37 °C, and followed by 15 min at 80 °C to inactivate ExoSAP. This method was simpler and much faster than ethanol precipitation.

Data analysis

DNA sequencing was performed by the Macrogen service centre (https://dna.macrogen.com). The final length was estimated between 1080 – 1117 bp in cyt b and 352 – 602 bp in S7 in different species, according to the sequence quality and presence of indels in S7. Shorter length of S7 sequences was due to presence of indels.

Obtained Cytochrome b and S7 sequences were checked manually in *Chromas v2.6.4* (http://technelysium.com.au/wp/chromas/) and aligned in *Bioedit v7.2.6.1* (Hall, 1999). The appropriate model of nucleotide substitutions was determined using *jModelTest v2.1.9* (Darriba et al., 2015). Model selection was based on Akaike information criterion (AIK) and Bayesian information criterion (BIC).

DnaSP v6.11.01 (http://www.ub.edu/dnasp/; (Rozas, 2009)) was used to analyze DNA polymorphism. Haplotype diversity (Hd), nucleotide diversity (π), number of sequenced

specimens (Ns), number of segregating sites (S) and number of haplotypes (Nh) are parameters describing genetic variability. Further, Fu and Li's and Tajima's tests of neutrality were performed to reveal whether the sequences evolved under random processes or were influenced by selection, introgression, demographic changes or other non-random processes; the results of these tests can point to possible bottlenecks and population expansion.

To evaluate the amount of genetic structure within and between populations, AMOVA (Analysis of molecular variance) was performed using *ARLEQUIN v3.5* (Excoffier and Lischer, 2010). It estimates population differentiation with the use of individual haplotypes and their frequency in the selected populations. This further enables calculation of fixation indices, such as F_{ST} and population pairwise F_{STs}. F_{ST} expresses the degree of genetic distance between the individual populations. The values range from 0, when the frequencies of alleles in the compared populations are the same, to 1, when the allele is fixed in one population and compared populations share no genetic diversity.

For converting formats of alignment data with the online program *ALTER* (http://www.sing-group.org/ALTER/; (Glez-Peña et al., 2010)) was used. The haplotype networksfor both mitochondrial and nuclear markers were constructed with *TCS v1.21* (Clement et al., 2000). The network estimation implemented in TCS is Statistical Parsimony. It was used under a 95% connection limit, with exception of complex *Zebrus* - *Millerigobius* where connection limit was fixed at 60 steps (Templeton et al., 1992), in order to show the distance between the outlining haplotypes (see Results). For the visualisation of the haplotype networks, a web-based program *tcsBU* (Santos et al., 2015) was used.

Program *PHASE v2.1.1* (http://stephenslab.uchicago.edu/phase/download.html) was used to reconstruct haplotypes (to phase data, i.e. heterozygotic positions) of nuclear gene S7. Only sequences which were phased with the probability higher than 0.7 were used, the remaining ones were not used for further analyses. The phased data were used for haplotype networks, DAPC, Diversity measures, AMOVA, Pairwise F_{STs}, Genetic distances (uncorrected p-distances) and Geneland analysis.

Discriminant analysis of principal components (DAPC) was applied using the package *Adegenet v2.1.1* (Jombart, 2008) for software *R v3.5* in order to investigate the population structure. The genotype data is firstly processed using a principal component analysis (PCA). Then discriminant analysis (DA) is performed for identification of genetic clusters using k-means clustering according to the Bayesian Information Criterion (BIC). It minimizes variance within groups whereas optimizes differences between groups. In the resulting scatterplots, the dots represent individuals and ellipses correspond to geographical populations.

Past population demography was inferred using the linear Bayesian skyline plot (BSP) model, implemented in *BEAST v1.8.4* (Bouckaert et al., 2014). It allows observing fluctuations of population abundances from the recent to the coalescence of the examined sequences and is expressed graphically. Analysis was conducted under Bayesian coalescent method with corresponding substitution model for each species and marker and using a strict

molecular clock. Dating was not performed, as no relevant dating point or fossil was available, so the x-axis shows the time in mutation units per nucleotide position. Simulations ran for 100 million MCMC steps with sampling every 10 000 generations. Results from 3 independent runs were combined for each species and marker using LogCombiner and burn-in was set to 20 million iterations in each run. *TRACER v1.7.0* (Rambaut et al., 2018) was used to assess the Bayesian skyline plots and convergence of chains.

Software - *Geneland v4.0.8* (Guillot et al., 2005) was used in program *R v3.4* to analyse the spatial genetic architecture, i.e., to display the distribution of genotypes according to their geographical origin. It performs Bayesian clustering based on genotype frequencies, but also takes into account the spatial origin of individuals given by GPS coordinates. Thus it helps to detect and localize genetic discontinuities along the sampling area. Number of MCMC iterations was set to 1 000 000, saving each 100th generation, with uncorrelated frequency model. Maximum number of set populations ranged always between 7 and 12 and it run five times independently. The posterior probability of the subpopulation membership for the most supported run was computed using a 20% burn-in. Although this work is focused only on marine species, Geneland includes also land in inferred distribution.

The results revealed a higher genetic diversity in two cases (*Chromogobius zebratus* and *Zebrus – Millerigobius* complex) and revealed the existence of several clades within the data. Therefore, I analysed phylogenetic relationships among the two lineages where these species complexes belong (genus *Chromogobius* and *Zebrus – Millerigobius* complex), based on cyt b using two reconstructions. Bayesian inference (BI) was conducted in *Mr. Bayes v3.2.6* (Ronquist et al., 2012). Two runs were performed simultaneously with Markov Chain Monte Carlo (MCMC) sampling set for at least 1 000 000 generations. Trees were sampled every 100th generations and ran until the standard deviation of split frequencies was < 0.01. The first 25 % of trees of both runs were discarded as burn-in and remaining trees were used to construct a 50 % majority-rule consensus tree. For rooting of phylogenetic trees, sequence of *Corcyrogobius liechtensteini* was used as outgroups for the genus *Chromogobius*, and *Gammogobius steinitzi* for the clade *Zebrus - Millerigobius*.

Second phylogenetic analysis applied, Maximum Likelihood (ML), was conducted in *MEGA v6* (http://www.megasoftware.net/). Support for each node was assessed with 1000 bootstrap replications and corresponding substitution model was set to compute the tree. Both phylogenetic trees were then visualized in *FigTree v1.4.3* (http://tree.bio.ed.ac.uk/software/figtree/) and graphically modified with program *InkScape v0.92.3*. Furthermore, program *MEGA 6* was also used to calculate genetic distances (uncorrected p-distances) between and within populations of each species.

Results

Corcyrogobius liechtensteini

A total of 48 sequences of specimens of *Corcyrogobius liechtensteini* of 1080 bp length were processed for mitochondrial gene cyt b, and 49 specimens for nuclear gene S7 (with 352 bp sequence fragment lengths). For S7, data were phased and used for subsequent analyses except of Bayesian Skyline plot, where unphased data were used. The best-fit substitution model for cyt b was General Time Reversible (GTR) calculated by both Akaike and Bayesian criterion. Hasegawa-Kishino-Yano with invariant sites (HKY+I) was the best model for S7 calculated by AIC and Kimura 2-parameter (K80) with invariant sites calculated by BIC. For cyt b, haplotype diversity (Hd = 0.662) was high, while nucleotide diversity low (π = 0.00155), which indicates recent population expansion. This was also suggested by negative values of neutrality tests (Tajima's D, Fu and Li's), which were all significant (see Tab. 6). The results of S7 showed very high haplotype diversity (Hd = 0.934) as well as nucleotide diversity (π = 0.01590), which suggests that it is large population with stable effective size. The results of neutrality tests were positive for S7 and significant in case of Fu and Li's F and D (see Tab. 7). The polymorphism of S7 marker was actually higher, but due to presence of multiple indels in the dataset, the alignment was shortened to the start of first indel. In addition, 26 sequences were not used for further analyses, because of low reliability of phased results.

Table 6: Diversity measures of *C. liechtensteini* for cyt b. Ns - number of sequences, S - number of segregating sites, Nh - number of haplotypes, Hd - haplotype diversity, π - nucleotide diversity, Fu & Li‘s F, Fu & Li’s D and Tajima’s D. Statistical significance of Fu & Li’s F and D **, $p < 0.02$ and Tajima’s D **, $p < 0.01$.

Species	Ns	S	Nh	Hd	π	Fu & Li's F	Fu & Li's D	Tajima's D
Corcyrogobius liechtensteini	48	29	19	0.662	0.00155	**-4,03478****	**-3,89046****	**-2,48191****

Table 7: Diversity measures of *C. liechtensteini* for S7. Ns - number of sequences (alleles), S - number of segregating sites, Nh - number of haplotypes, Hd - haplotype diversity, π - nucleotide diversity, Fu & Li‘s F, Fu & Li’s D and Tajima’s D. Statistical significance of Fu & Li’s F *, $p < 0.05$ and Fu & Li’s D **,$p < 0.02$.

Species	Ns	S	Nh	Hd	π	Fu & Li's F	Fu & Li's D	Tajima's D
Corcyrogobius liechtensteini	72	19	26	0.934	0.01590	**1.84113**	**1.69471**	1.28363

In AMOVA analysis six localities (Greece, Sicily, Croatia, France, Cyprus south (S), Cyprus north (N)) were included (see Fig. 4). Results for both cyt b and S7 indicate absence of genetic structure among the populations. All genetic variation is explained by the

variability within the populations. Zero F_{ST} index assessed for cyt b indicates panmictic population. Comparisons of pairwise F_{STs} for cyt b between six localities had values from 0 to 0.818 (France with Croatia), which was the only positive value close to one. The results suggest that only populations from France, Croatia and Cyprus S differ between each other. The rest of the localities have zero values or values close to zero what indicates no differentiation (see Tab. 8 and 9). For S7, F_{ST} index reached value of low differentiation (0.04092). Pairwise differences for S7 ranged between 0 and 0.466 (Sicily with France). Generally, France differed the most from the other localities, however, there was only one specimen from this locality in dataset (see Fig. 10 and 11).

Table 8 and 9: AMOVA and pairwise F_{STs} for *C. liechtensteini* for cyt b.
Values with statistical significance $p < 0.05$/number of pairs are shown in bold.

AMOVA	**Degrees of freedom**	**Variance components**	**% of variation**	**F_{ST}**
among populations	4	-0.01155	-1.38	0
within populations	43	0.84610	101.38	

pairwise F_{STs}	Greece	Sicily	Croatia	France	Cyprus S	Cyprus N
Greece	0.000					
Sicily	**0.000**	**0.000**				
Croatia	**0.000**	**0.002**	**0.000**			
France	**0.000**	**0.000**	0.818	**0.000**		
Cyprus S	**0.000**	**0.006**	0.067	0.091	**0.000**	
Cyprus N	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.000

Table 10 and 11: AMOVA and pairwise F_{STs} for *C. liechtensteini* for S7.
Values with statistical significance $p < 0.05$/number of pairs are shown in bold.

AMOVA	**Degrees of freedom**	**Variance components**	**% of variation**	**F_{ST}**
among populations	4	0.11571	4.09	0.04092
within populations	67	2.71211	95.91	

pairwise F_{STs}	Croatia	France	Greece	Sicily	Cyprus S	Cyprus N
Croatia	0.000					
France	0.282	0.000				
Greece	**0.000**	0.338	0.000	0.000		
Sicily	**0.015**	0.466	**0.000**	0.000	0.000	
Cyprus S	0.069	0.447	**0.018**	**0.041**	0.000	
Cyprus N	**0.000**	0.388	**0.000**	**0.000**	**0.000**	0.000

Haplotype network for cyt b revealed one dominant central haplotype shared between five geographically distant areas (Croatia, Sicily, Greece, Cyprus S and Cyprus N). There is one more, small shared haplotype, and several unique haplotypes, in most cases very similar to the central one (see Fig. 20). In all localities more than one haplotype was revealed, with the exception of France, where only one individual was available. The result of DAPC shows

that the haplotype from France is different from the remaining ones, with the exception of Sicily (see Fig. 21). Interestingly, result of nuclear gene S7 shows 16 haplotypes shared between the localities, much greater variability and no genetic structure (see Fig. 22). The result of DAPC for S7 reveals the interconnection of all localities, see Fig. 23.

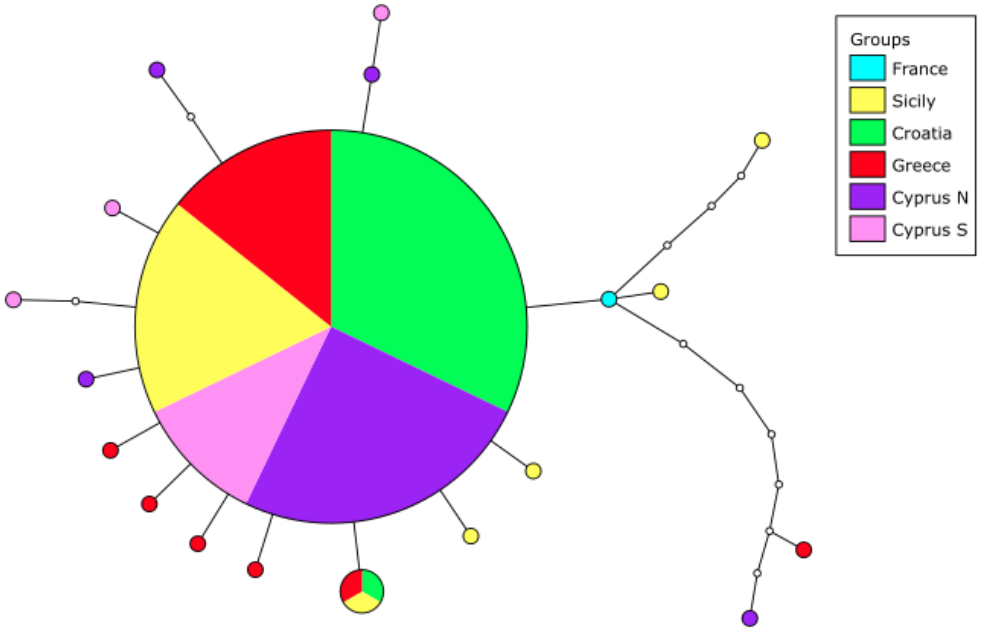

Figure 20:
Haplotype network for *C. liechtensteini* for cyt b. The colours correspond to localities described in legend and area of the circles is proportional to each haplotype frequency.

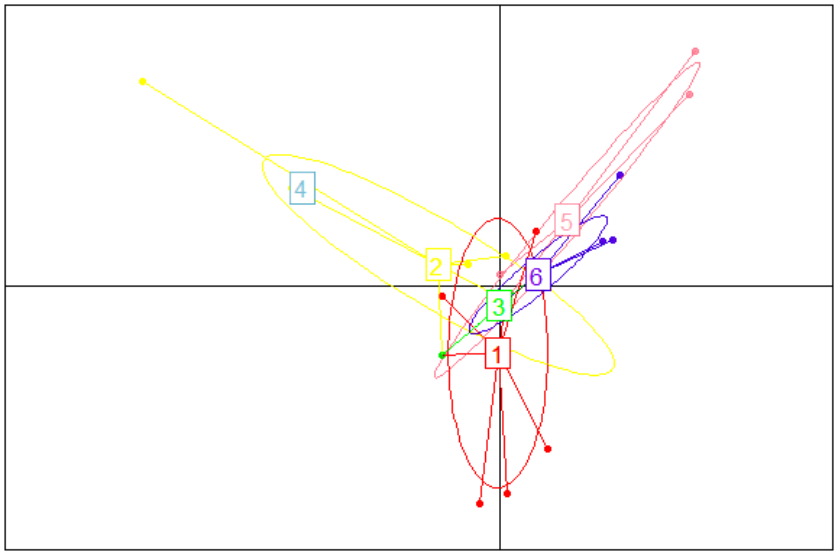

Figure 21:
Discriminant Analysis of Principal Components (DAPC) for *C. liechtensteini* for cyt b. Dots refer to haplotypes and inertia ellipses to groups. No ellipse means only one haplotype at the given locality. Colours of populations are identical to those used in the haplotype network: 1 (red) Greece, 2 (yellow) Sicily, 3 (green) Croatia, 4 (light blue) France, 5 (pink) Cyprus N, 6 (purple) Cyprus S.

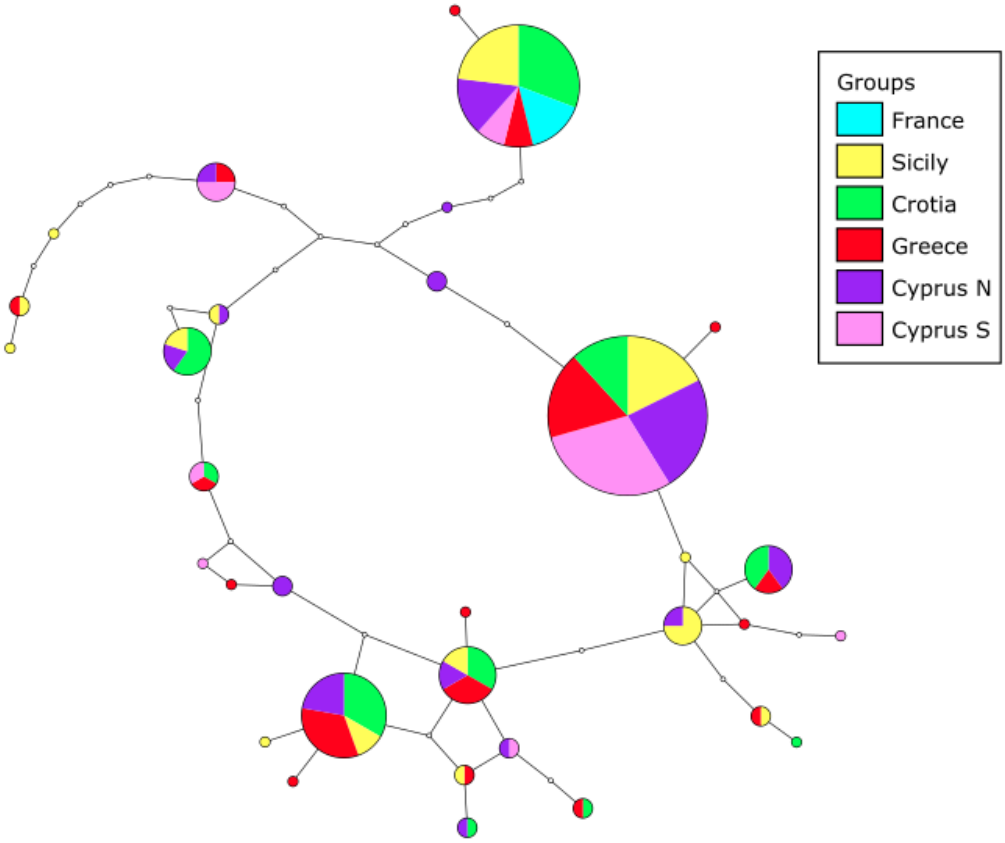

Figure 22:
Haplotype network for *C. liechtensteini* for S7. The colours correspond to localities described in legend and area of the circles is proportional to each haplotype frequency.

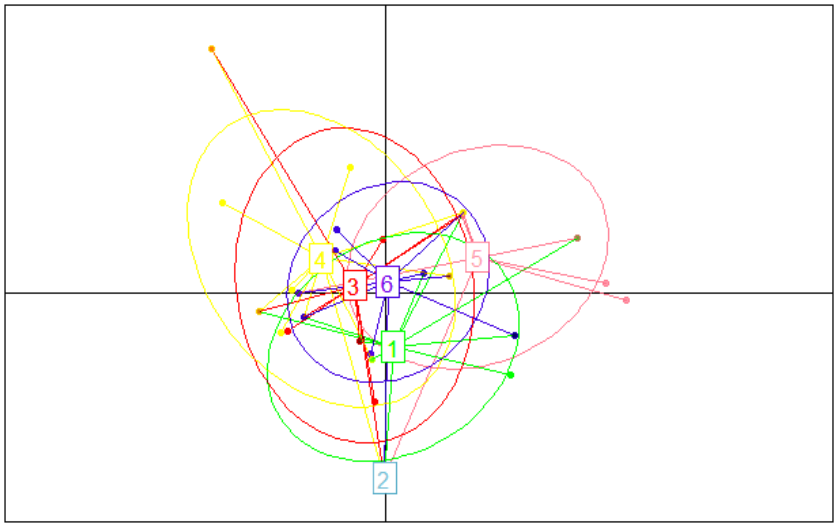

Figure 23:
Discriminant Analysis of Principal Components (DAPC) for *C. liechtensteini* for S7. Dots refer to haplotypes and inertia ellipses to groups. No ellipse means only one haplotype at the given locality. Colours of populations are identical to those used in the haplotype network: 1 (green) Croatia, 2 (light blue) France, 3 (red) Greece, 4 (yellow) Sicily, 5 (pink) Cyprus N, 6 (purple) Cyprus S.

Graphical record of population dynamics was estimated using the method of Bayesian Skyline plot. The substitution model used was GTR for cyt b, and HKY + I model was used for S7 as the most similar to the assessed one. The Bayesian Skyline plot for cyt b shows constant population in the past with population growth and again steady population in present (see Fig. 24) whereas graph for S7 shows constant population size with recent decline (see Fig. 25).

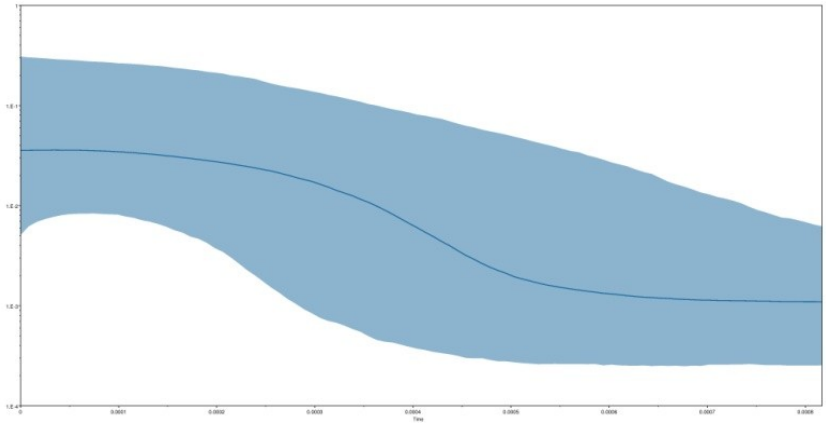

Figure 24:
Bayesian skyline plot for *C. liechtensteini* for cyt b. The course of the graph illustrates fluctuation of the population size from recent to the coalescence (left to the right). Y-axis stands for effective population size and x-axis for time scale in units of mutation on a nucleotide position. Blue middle line shows the median estimate and the outer lines of blue area represent 95% confidence interval.

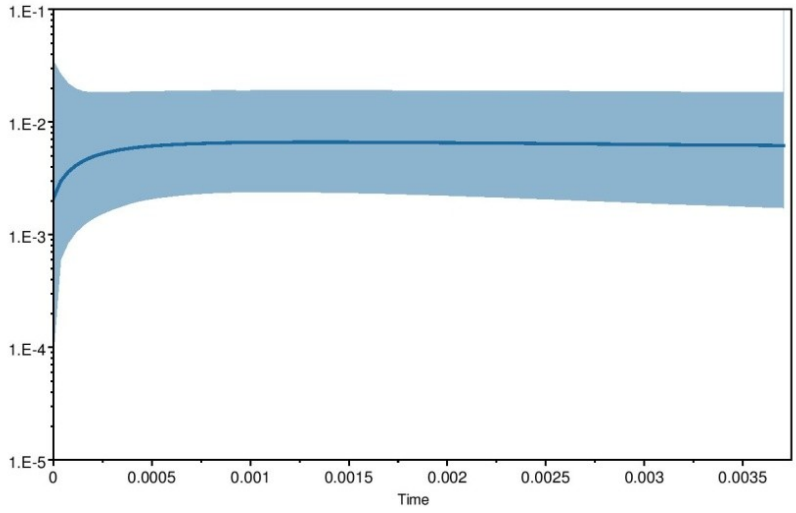

Figure 25:
Bayesian skyline plot for *C. liechtensteini* for S7. The course of the graph illustrates fluctuation of the population size from recent to the coalescence (left to the right). Y-axis stands for effective population size and x-axis for time scale in units of mutation on a nucleotide position. Blue middle line shows the median estimate and the outer lines of blue area represent 95% confidence interval.

The result of the Geneland for cyt b shows that analysed samples of *C. liechtensteini* belong to one population (Fig 26). This corresponds with other results for cyt b, which suggested that the population is panmictic. The result of the analysis for S7 showed that distribution of *C. liechtensteini* is divided into two populations. One single population comprises Cyprus and rest of the localities belong to the second population (see Fig. 27).

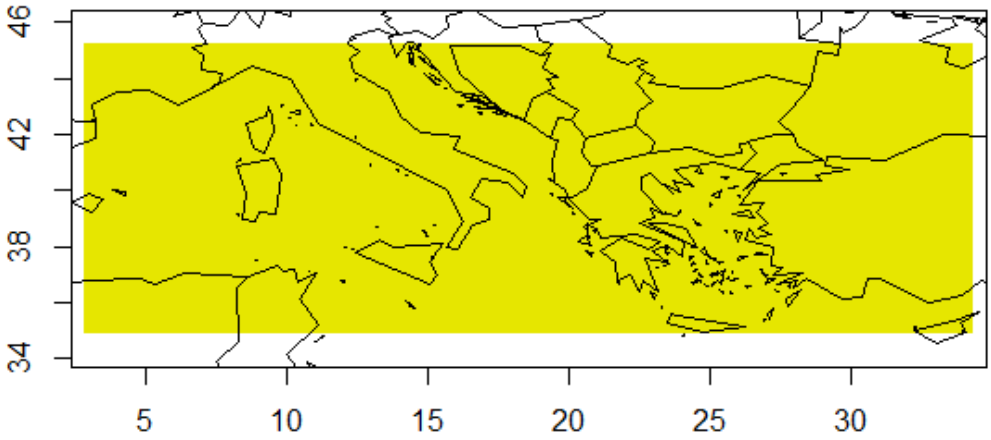

Figure 26:
Spatial distribution of *C. liechtensteini* for cyt b by Geneland. Y-axis refers to geographical latitude and x-axis longitude.

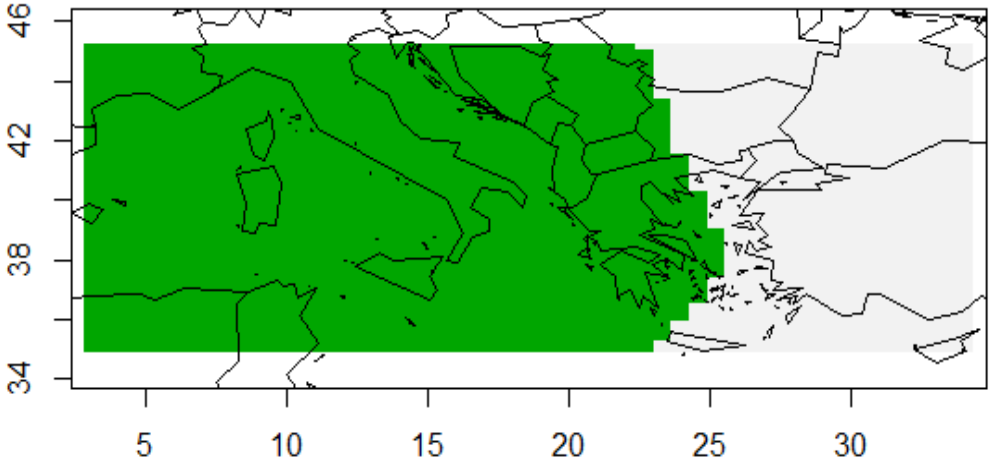

Figure 27:
Spatial distribution of populations of *C. liechtensteini* for S7 by Geneland. Each colour corresponds to one sub-population. Y-axis refers to geographical latitude and x-axis geographical longitude.

Mean genetic distances (uncorrected p-distances) for cyt b between groups (localities) are very low (0.1 - 0.2%). That points to lack of geographic subdivisions of *C. liechtensteini*. The genetic distances within localities were in the similar range of magnitude (see Tab. 12). Interestingly, genetic distances for S7 (both between and within groups) are one range of magnitude higher (1.4 - 2.3%). The sample from France was slightly more divergent from the rest of the populations (see Tab.13).

Table 12: Genetic distances for *C. liechtensteini* for cyt b (uncorrected p-distances). Db - distance between groups, Dw - distance within group, n/c - not compared (only one specimen in dataset).

Db	Greece	Sicily	Croatia	France	Cyprus S	Cyprus N	**Dw**
Greece							0.0022
Sicily	0.002						0.0018
Croatia	0.001	0.001					0.0002
France	0.002	0.001	0.001				n/c
Cyprus S	0.002	0.002	0.001	0.002			0.0015
Cyprus N	0.002	0.002	0.001	0.002	0.002		0.0020

Table 13: Genetic distances for *C. liechtensteini* for S7 (uncorrected p-distances). Db - distance between groups, Dw - distance within group, n/c - not compared (only one specimen in dataset).

Db	Croatia	France	Greece	Sicily	Cyprus S	Cyprus N	**Dw**
Croatia							0.0162
France	0.018						n/c
Greece	0.017	0.022					0.0183
Sicily	0.016	0.023	0.016				0.0149
Cyprus S	0.016	0.021	0.017	0.015			0.0144
Cyprus N	0.016	0.021	0.017	0.015	0.015		0.0159

Gobius cruentatus

A total of 37 specimens were processed for mitochondrial gene cyt b (alignment length 1117 bp) and 35 specimens for nuclear gene S7 (512 bp fragment lengths). The dataset for S7 was phased and phased data were then used for calculating diversity measures and constructing haplotype network. Bayesian skyline plot and graphical spatial subdivision of population by Geneland was not performed due to very low polymorphism of S7 dataset. The actual variability of the dataset was higher provided by presence of indels, however, the dataset was shortened to the beginning of the first indel. The best-fit substitution model for cyt b calculated by AIC was General Time Reversible with invariant sites (GTR+I) and Hasegawa-Kishino-Yano with invariant sites (HKY+I) calculated by BIC. Felsenstein 1981 (F81) model was best model for S7 calculated by both AIC and BIC. For cyt b, haplotype diversity (Hd = 0.985) was much greater than nucleotide diversity (π = 0.00656) what indicates recent population expansion. This was also confirmed by negative values of neutrality tests (Tajima's D, Fu and Li's), where only Fu and Li's D was significant (see Tab. 14). For S7, haplotype diversity (Hd = 0.185) was also greater than nucleotide diversity (π = 0.00037), however both values were very low. The results of neutrality tests for S7 were again negative but not statistically significant (see Tab. 15).

Table 14: Diversity measures of *G. cruentatus* for cyt b. Ns - number of sequences, S - number of segregating sites, Nh - number of haplotypes, Hd - haplotype diversity, π - nucleotide diversity, Fu & Li‘s F, Fu & Li’s D and Tajima’s D. Statistical significance of Fu & Li’s D *, $p < 0.05$.

Species	Ns	S	Nh	Hd	π	Fu & Li's F	Fu & Li's D	Tajima's D
Gobius cruentatus	37	46	30	0.985	0.00656	-2.45860	**-2.50090***	-1.25290

Table 15: Diversity measures of *G. cruentatus* for S7. Ns - number of sequences, S - number of segregating sites, Nh - number of haplotypes, Hd - haplotype diversity, π - nucleotide diversity, Fu & Li‘s F, Fu & Li’s D and Tajima’s D.

Species	Ns	S	Nh	Hd	π	Fu & Li's F	Fu & Li's D	Tajima's D
Gobius cruentatus	70	2	3	0.185	0.00037	-1.11813	-0.98333	-0.90551

In AMOVA analysis seven localities (Sicily, Croatia, Montenegro, Spain, Portugal, France, Cyprus north (N)) were included (see Fig. 6). Most of the genetic variability (80%) was due to variance within populations. F_{ST} index (0.20449) was significant and reached a value of high differentiation. Comparisons of pairwise F_{STs} between seven localities had values from 0 to 0.725 (Cyprus N with Spain), which means a relatively large scale. The highest differentiation was detected between the most eastern locality, Cyprus, and three most western localities, Spain, Portugal and France, however, the values were not significant (see Tab. 16 and 17).

Table 16 and 17: AMOVA and pairwise F_{STs} for *G. cruentatus* for cyt b.
Values with statistical significance $p < 0.05$/number of pairs are shown in bold.

AMOVA	**Degrees of freedom**	**Variance components**	**% of variation**	**F_{ST}**
among populations	5	0.78382	20.45	**0.20449**
within populations	31	3.04921	79.55	

pairwise F_{STs}	Sicily	Croatia	Montenegro	Spain	Portugal	France	Cyprus N
Sicily	0.000						
Croatia	**0.000**	0.000					
Montenegro	**0.000**	**0.000**	0.000				
Spain	0.407	0.223	0.298	0.000			
Portugal	0.334	0.131	**0.030**	0.090	0.000		
France	0.351	0.151	**0.028**	0.147	**0.000**	0.000	
Cyprus N	0.053	0.057	0.109	0.725	0.645	0.571	0.000

From the haplotype network for cyt b it can be seen that only three haplotypes are shared and most haplotypes are unique (see Fig. 28). In contrary, there was almost no polymorphism in S7, there was one big haplotype shared among all localities and two small ones (see Fig. 30). Discriminant analysis for cyt b separated subpopulation from Cyprus from Montenegro, France, Portugal and Spain the remaining subpopulations were interconnected (see Fig. 29).

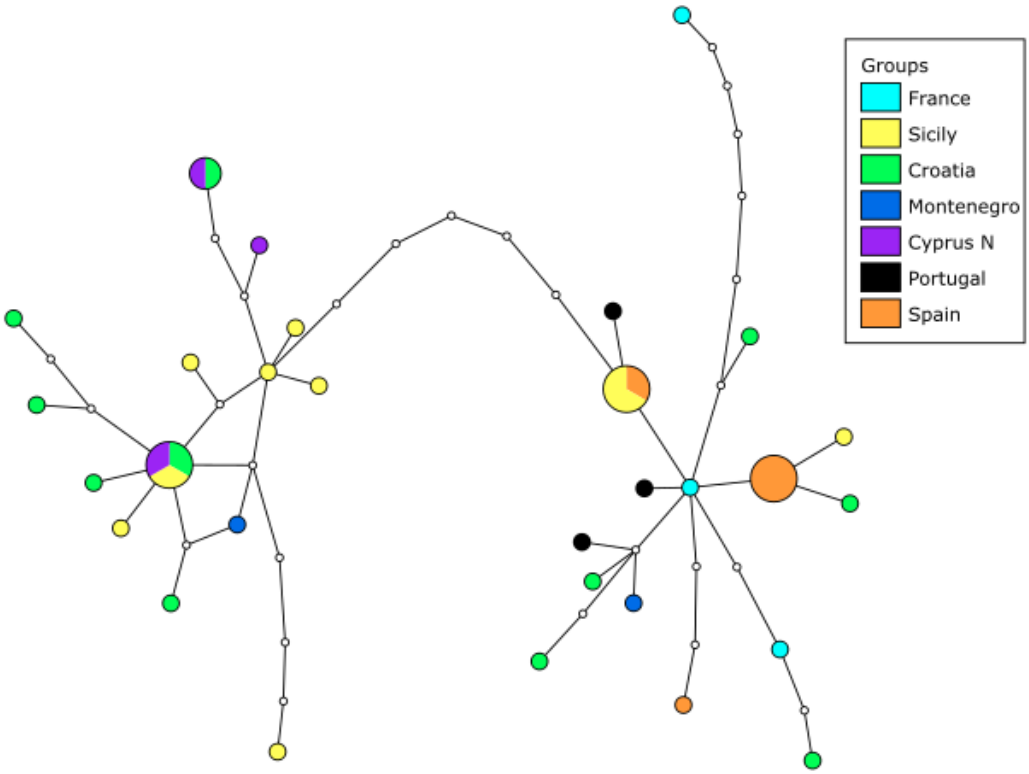

Figure 28:
Haplotype network for *G. cruentatus* for cyt b. The colours correspond to localities described in legend and area of the circles is proportional to each haplotype frequency.

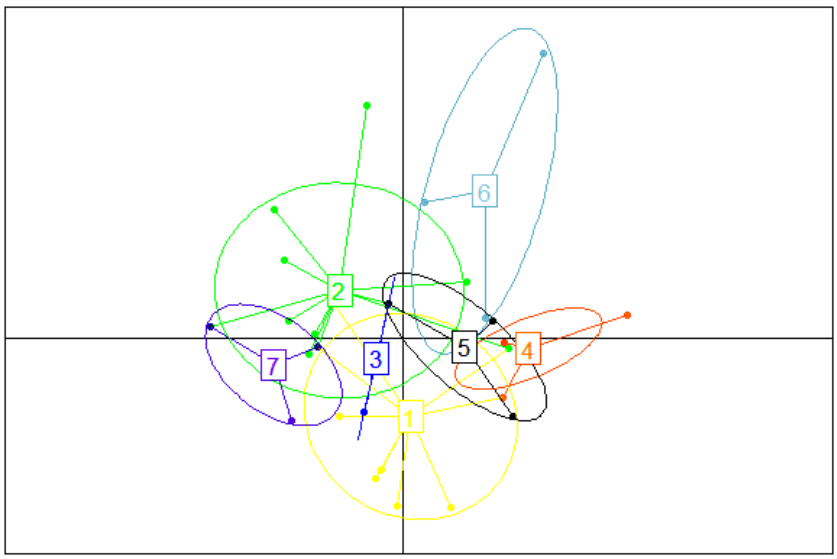

Figure 29:
Discriminant Analysis of Principal Components (DAPC) for *G. cruentatus* for cyt b. Dots refer to haplotypes and inertia ellipses to groups. Colours of populations are identical to those used in the haplotype network: 1 (yellow) Sicily, 2 (green) Croatia, 3 (dark blue) Montenegro, 4 (orange) Spain, 5 (black) Portugal, 6 (light blue) France, 7 (purple) Cyprus S.

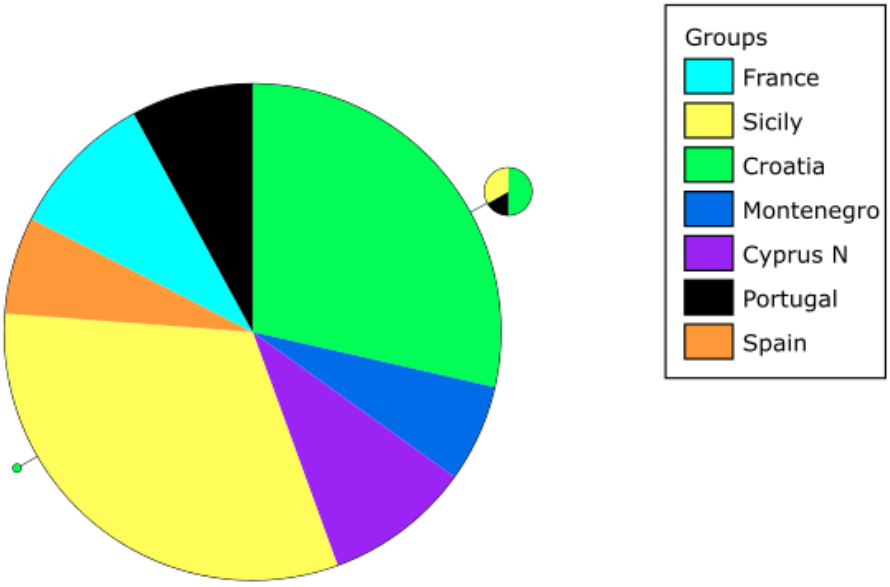

Figure 30:
Haplotype network for *G. cruentatus* for S7. The colours correspond to localities described in legend and area of the circles is proportional to each haplotype frequency.

The substitution model used for Bayesian skyline plot construction was HKY + I. The Bayesian skyline plot shows gradual growth in the past with indicated constant population in recent (see Fig. 31).

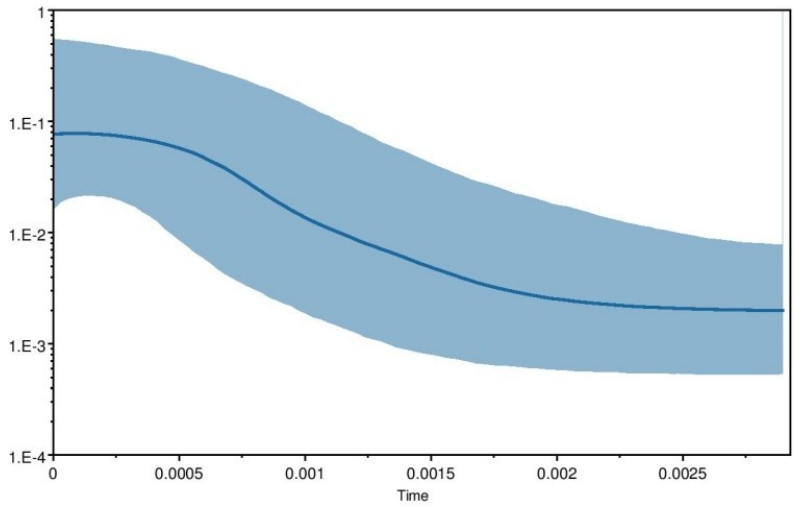

Figure 31:
Bayesian skyline plot for *G. cruentatus* for cyt b. The course of the graph illustrates fluctuation of the population size from recent to the coalescence (left to the right). Y-axis stands for effective population size and x-axis for time scale in units of mutation per nucleotide position. Blue middle line shows the median estimate and the outer lines of blue area represent 95% confidence interval.

By Geneland spatial analysis, three populations were revealed, eastern, western and central (actually, no samples were used from the central region), see Fig 32.

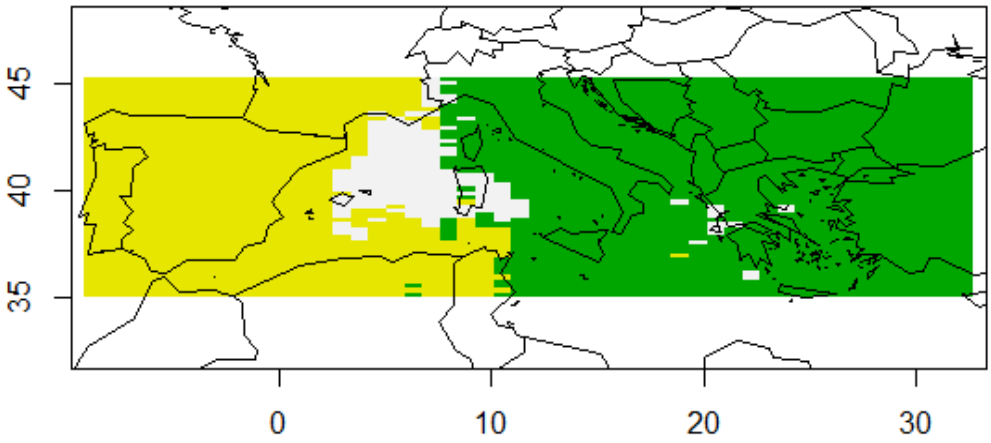

Figure 32:
Spatial distribution of populations of *G. cruentatus* for cyt b by Geneland. Each colour corresponds to one sub-population . Y-axis refers to geographical latitude and x-axis geographical longitude.

Mean genetic distances (uncorrected p-distances) were between groups were very low. The largest divergence (1.1%) was determined between Cyprus N and France and the smallest (0.3%) between Portugal and Spain. Intraspecific distances were in the same order of magnitude, with the highest value for Montenegro (see Tab. 18).

Table 18: Genetic distances for *G. cruentatus* for cyt b (uncorrected p-distances). Db - distance between groups, Dw - distance within group.

Db	Sicily	Croatia	Montenegro	Spain	Portugal	France	Cyprus N	**Dw**
Sicily								0.0052
Croatia	0.006							0.0079
Montenegro	0.006	0.007						0.0100
Spain	0.007	0.007	0.006					0.0020
Portugal	0.007	0.007	0.006	0.003				0.0030
France	0.008	0.008	0.007	0.004	0.004			0.0054
Cyprus N	0.005	0.007	0.007	0.009	0.009	0.011		0.0036

Gobius geniporus

A total of 63 specimens were processed for mitochondrial gene cyt b (alignment length 1117 bp) and 68 specimens for nuclear gene S7 (555 bp fragment lengths). The dataset for S7 was phased and phased data were then used for calculating diversity measures and constructing haplotype network, due to practically no polymorphism in S7 dataset. The variability was actually somewhat higher, provided by the presence of indels, however the dataset was shortened to the first indel. Then, the rest of the analyses were done only for cyt b. The best-fit substitution model for cyt b assessed both by AIC and BIC was General Time Reversible but for AIC with invariant sites and gamma distribution (GTR+I+G) and for BIC only with invariant sites (GTR+I). Felsenstein 1981 (F81) model was the best model for S7 assessed by both AIC and BIC. For cyt b, haplotype diversity (Hd = 0.969) was high, while nucleotide diversity low (π = 0.00440) what indicates recent population expansion. This was also confirmed by negative values of neutrality tests (Tajima's D, Fu and Li's), which were all significant (see Tab. 19). For S7, values of haplotype (Hd = 0.058) and nucleotide diversity (π = 0.00010) were extremely low. The results of neutrality tests were again negative but not statistically significant (see Tab. 20).

Table 19: Diversity measures of *G. geniporus* for cyt b. Ns - number of sequences, S - number of segregating sites, Nh - number of haplotypes, Hd - haplotype diversity, π - nucleotide diversity, Fu & Li‘s F, Fu & Li’s D and Tajima’s D. Statistical significance of Fu & Li‘s F and D **,
$p < 0.02$ and Tajima’s D *, $p < 0.05$.

Species	Ns	S	Nh	Hd	π	Fu & Li's F	Fu & Li's D	Tajima's D
Gobius geniporus	63	50	42	0.969	0.00440	**-3.38338***	**-3.45810***	**-1.80005***

Table 20: Diversity measures of *G. geniporus* for S7. Ns - number of sequences, S - number of segregating sites, Nh - number of haplotypes, Hd - haplotype diversity, π - nucleotide diversity, Fu & Li‘s F, Fu & Li’s D and Tajima’s D.

Species	Ns	S	Nh	Hd	π	Fu & Li's F	Fu & Li's D	Tajima's D
Gobius geniporus	136	2	3	0.058	0.00010	-1.36934	-1.14143	-1.22206

In AMOVA analysis samples from seven localities (Sicily, Croatia, Montenegro, France, Cyprus north (N), Cyprus south (S), Greece) were included (see Fig. 8). Most of the genetic variability (66%) was due to variance within populations however, variation among populations was higher than in other species. F_{ST} index (0.33533) was significant and reached value of very high differentiation. Comparisons of pairwise F_{STs} between seven localities had values from 0 (Montenegro with Croatia) to 0.506 (Cyprus N with Sicily). Almost all localities considerably differ between each other, but the values are not statistically significant. The only significant values were between 0 and 0.036, suggesting no differentiation between Montenegro and Croatia, Cyprus N and Cyprus S, and Cyprus N and Greece (see Tab. 21 and 22).

Table 21 and 22: AMOVA and pairwise F_{STs} for *G. geniporus* for cyt b.
Values with statistical significance $p < 0.05$/number of pairs are shown in bold.

AMOVA	**Degrees of freedom**	**Variance components**	**% of variation**	**F_{ST}**
among populations	5	0.88287	33.53	**0.33533**
within populations	57	1.74993	66.47	

pairwise F_{STs}	Sicily	Croatia	Montenegro	France	Cyprus N	Cyprus S	Greece
Sicily	0.000						
Croatia	0.382	0.000					
Montenegro	0.451	**0.000**	0.000				
France	0.193	0.197	0.279	0.000			
Cyprus N	0.506	0.324	0.349	0.408	0.000		
Cyprus S	0.505	0.405	0.430	0.383	**0.036**	0.000	
Greece	0.419	0.136	0.138	0.193	**0.004**	0.100	0.000

There is a certain geographical pattern in the genetic structure of *G. geniporus* for cyt b, since in the haplotype network individuals from Cyprus and Greece are grouped together (and moreover share two haplotypes), as well as those from Croatia and Montenegro (also sharing two haplotypes) and also from Sicily (see Fig. 33). There is a prevalence of unique haplotypes. Discriminant analysis clearly separates subpopulations from Sicily and France from all other subpopulations (see Fig. 34). Also sub-populations from Cyprus are separated from those from Croatia and Montenegro. In contrary, in S7, there is only one big haplotype shared among all localities, and two very small ones (see Fig. 35).

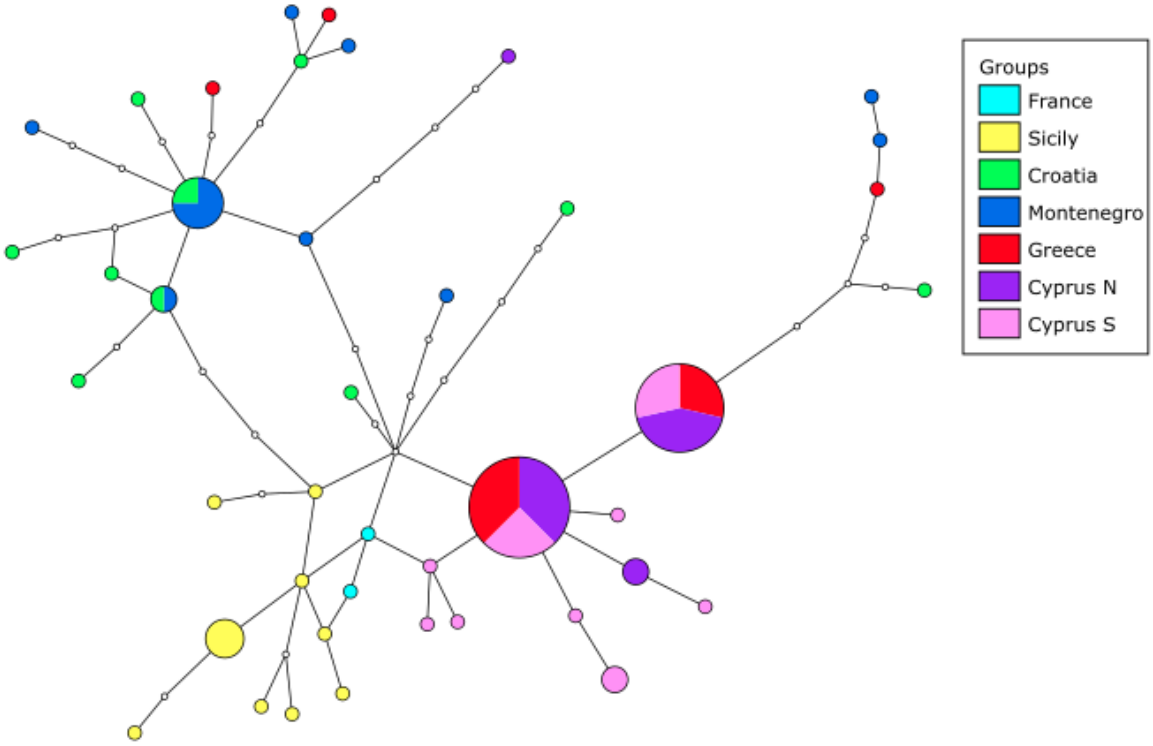

Figure 33:
Haplotype network for *G. geniporus* for cyt b. The colours correspond to localities described in legend and area of the circles is proportional to each haplotype frequency.

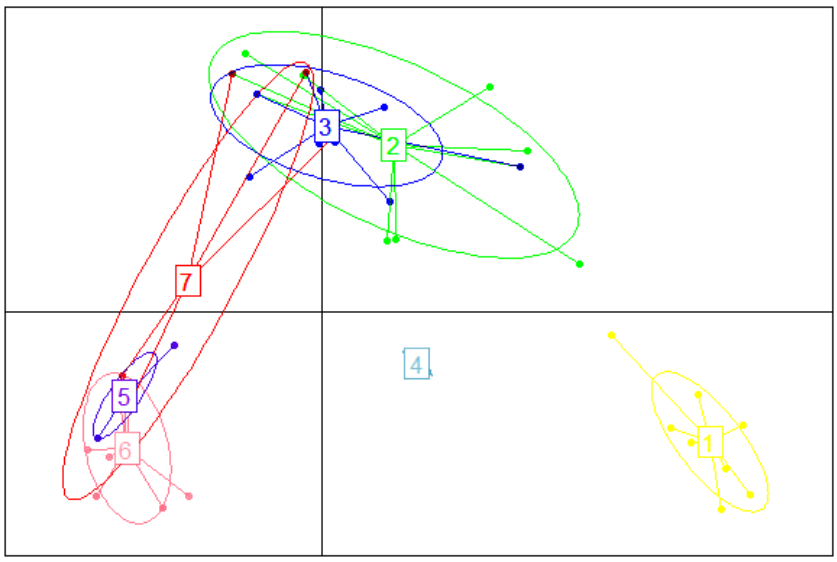

Figure 34:
Discriminant Analysis of Principal Components (DAPC) for *G. geniporus* for cyt b. Dots refer to haplotypes and inertia ellipses to groups. No ellipse means only one haplotype at the given locality. Colours of populations are identical to those used in the haplotype network: 1(yellow) Sicily, 2 (green) Croatia, 3 (dark blue) Montenegro, 4 (light blue) France, 5 (purple) Cyprus S, 6 (pink) Cyprus N, 7 (red) Greece.

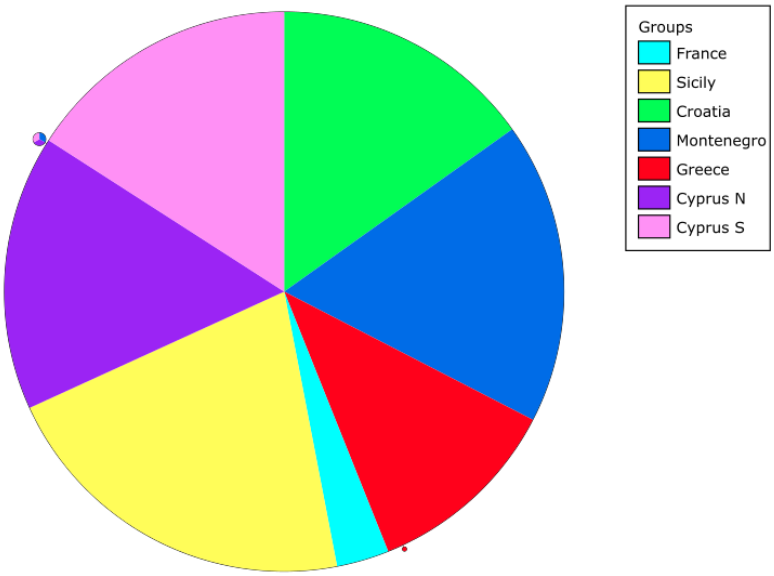

Figure 35:
Haplotype network for *G.geniporus* for S7. The colours correspond to localities described in legend and area of the circles is proportional to each haplotype frequency.

The substitution model used for constructing Bayesian Skyline plot was GTR + G. Bayesian Skyline plot for cyt b shows gradual growth with some oscillations (see Fig. 36).

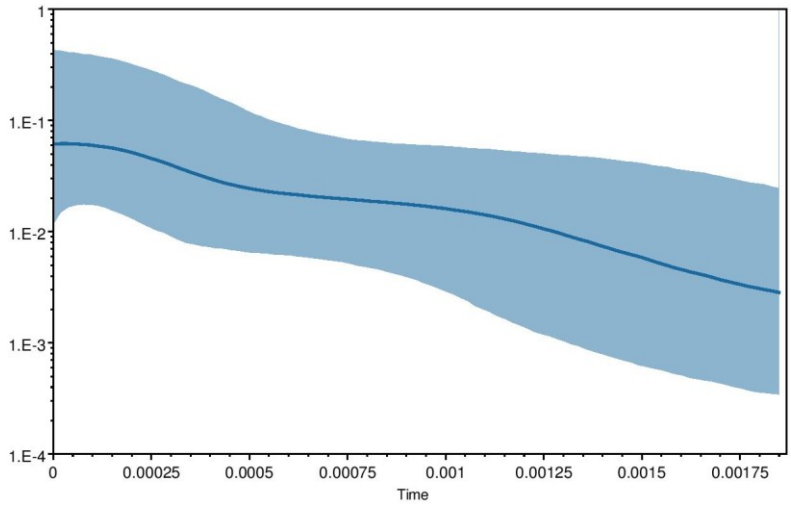

Figure 36:
Bayesian skyline plot for *G. geniporus* for cyt b. The course of the graph illustrates fluctuation of the population size from recent to the coalescence (left to the right).Y-axis stands for effective population size and x-axis for time scale in units of mutation on a nucleotide position. Blue middle line shows the median estimate and the outer lines of blue area represent 95% confidence interval.

The Geneland divided *G. geniporus* to three populations, from western, eastern and northern central Mediterranean, see Fig 37. This corresponds to the results of haplotype network.

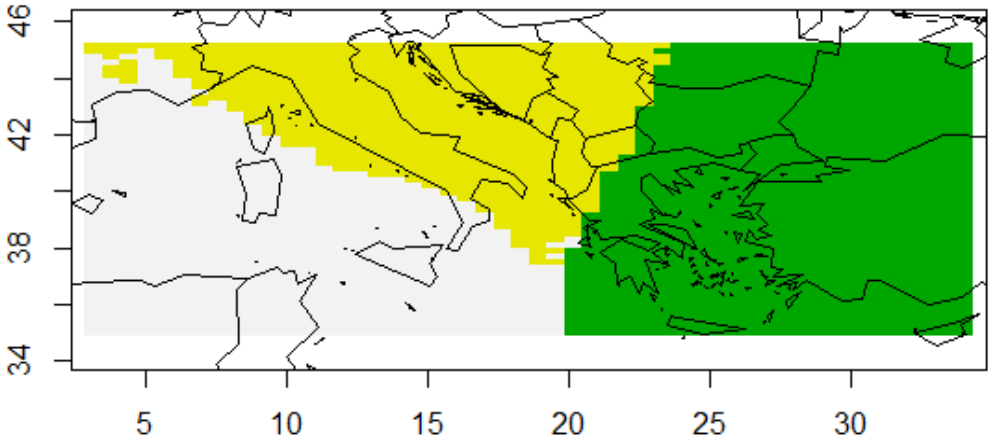

Figure 37:
Spatial distribution of populations of *G. geniporus* for cyt b by Geneland. Each colour corresponds to one sub-population . Y-axis refers to geographical latitude and x-axis geographical longitude.

Generally, p-distances are low and ranged between 0.2 and 0.6%. The highest divergence from other localities was for Croatian subpopulation, 0.6%, and it was with Sicily and Cyprus. The distance within the subpopulations ranged between 0.1 and 0.5% (see Tab. 23).

Table 23: Genetic distances for *G. geniporus* for cyt b (uncorrected p-distances). Db - distance between groups, Dw - distance within group.

Db	Sicily	Croatia	Montenegro	France	Cyprus N	Cyprus S	Greece	**Dw**
Sicily								0.0025
Croatia	0.006							0.0052
Montenegro	0.006	0.004						0.0040
France	0.003	0.005	0.005					0.0009
Cyprus N	0.005	0.006	0.005	0.003				0.0022
Cyprus S	0.005	0.006	0.005	0.003	0.002			0.0020
Greece	0.005	0.005	0.004	0.004	0.003	0.003		0.0036

Gobius incognitus

A total of 93 sequences of specimens of *Gobius incognitus* were processed for mitochondrial gene cyt b (1117 bp sequence length) and 71 specimens for nuclear gene S7 (602 bp sequence fragments). of the dataset for S7 was phased and phased data were then used for subsequent analyses except of Bayesian Skyline plot where unphased data were used. The best-fit substitution model for cyt b was General Time Reversible with invariant sites and gamma distribution (GTR+I+G) calculated by both AIC and BIC. Felsenstein 1981 (F81) model was the best model for S7 calculated by both AIC and BIC. For cyt b, haplotype diversity (Hd = 0.995) was high, hile nucleotide diversity low (π = 0.00750) what indicates recent population expansion. This was also confirmed by negative values of neutrality tests (Tajima's D, Fu and Li's), which were all significant (see Tab. 24). The results of S7 were similar and haplotype diversity (Hd = 0.839) was also greater than nucleotide diversity (π = 0.00306). The results of neutrality tests were again negative but not statistically significant (see Tab. 25). The polymorphism in S7 dataset was actually higher provided by presence of indels, however, the dataset was shortened to the beginning of the first indel. Moreover, 10 sequences were not used for further analyses, because the probability of phasing result was low.

Table 24: Diversity measures of *G. incognitus* for cyt b. Ns - number of sequences, S - number of segregating sites, Nh - number of haplotypes, Hd - haplotype diversity, π - nucleotide diversity, Fu & Li‘s F, Fu & Li’s D and Tajima’s D. Statistical significance of Fu & Li’s F and D **, $p < 0.02$ and Tajima’s D *, $p < 0.05$.

Species	Ns	S	Nh	Hd	π	Fu & Li's F	Fu & Li's D	Tajima's D
Gobius incognitus	93	105	81	0.995	0.00750	**-3.82446****	**-4.00472****	**-2.05719***

Table 25: Diversity measures of *G. incognitus* for cyt b. Ns - number of sequences, S - number of segregating sites, Nh - number of haplotypes, Hd - haplotype diversity, π - nucleotide diversity, Fu & Li‘s F, Fu & Li’s D and Tajima’s D.

Species	Ns	S	Nh	Hd	π	Fu & Li's F	Fu & Li's D	Tajima's D
Gobius incognitus	132	15	17	0.839	0.00306	-1.34648	-1.27331	-0.87869

In AMOVA analysis eight localities (Sicily, Greece, Montenegro, Croatia, France, Portugal, Cyprus north (N), Cyprus south (S)) were included (see Fig. 10). For cyt b, the majority (89%) of the genetic variance was found within populations. F_{ST} index (0.10993) was significant and reached value of medium differentiation. Pairwise F_{STs} for cyt b for eight localities range from 0 (Cyprus S with Cyprus N), the two geographically closest localities, to 0.249 (Cyprus N with Sicily), see Tab. 26 and 27. Also for S7 most of the genetic variation was found within the populations (94%), and F_{ST} index reached value of medium

differentiation (0.05570) with significance. Pairwise differences ranged between 0 and 0.276 (France with Montenegro). Montenegro appears to differentiate the most from the other localities, the exception is Portugal where there is surprisingly no difference between them (see Tab. 28 and 29). Only values showing no or very little differentiation were significant for both markers.

Table 26 and 27: AMOVA and pairwise F_{STs} for *G. incognitus* for cyt b.
Values with statistical significance $p < 0.05$/number of pairs are shown in bold.

AMOVA	**Degrees of freedom**	**Variance components**	**% of variation**	**F_{ST}**
among populations	6	0.46804	10.99	**0.10993**
within populations	86	3.78961	89.01	

pairwise F_{STs}	Sicily	Greece	Montenegro	Croatia	France	Portugal	Cyprus N	Cyprus S
Sicily	0.000							
Greece	0.083	0.000						
Montenegro	0.084	**0.009**	0.000					
Croatia	**0.019**	0.065	0.109	0.000				
France	0.059	0.061	0.055	0.103	0.000			
Portugal	**0.038**	**0.043**	0.052	**0.043**	**0.024**	0.000		
Cyprus N	0.249	0.093	0.147	0.215	0.210	0.152	0.000	
Cyprus S	0.229	0.077	0.119	0.198	0.198	0.141	**0.000**	0.000

Table 28 and 29: AMOVA and pairwise F_{STs} for *G. incognitus* for S7.
Values with statistical significance $p < 0.05$/number of pairs are shown in bold.

AMOVA	**Degrees of freedom**	**Variance components**	**% of variation**	**F_{ST}**
among populations	6	0.05188	5.57	**0.05570**
within populations	125	0.87956	94.43	

pairwise F_{STs}	Montenegro	Croatia	Greece	France	Portugal	Sicily	Cyprus N	Cyprus S
Montenegro	0.000							
Croatia	0.266	0.000						
Greece	0.169	**0.000**	0.000					
France	0.276	**0.000**	**0.000**	0.000				
Portugal	**0.000**	0.079	0.061	0.134	0.000			
Sicily	0.233	**0.000**	**0.000**	**0.000**	0.104	0.000		
Cyprus N	0.076	**0.001**	**0.000**	**0.035**	**0.000**	**0.006**	0.000	
Cyprus S	0.147	**0.000**	**0.000**	**0.000**	**0.035**	**0.026**	**0.000**	0.000

There is a great haplotype variability in cyt b (81 haplotypes), see haplotype network, Fig. 38. There are only four shared haplotypes and the vast majority of haplotypes are unique. No geographic pattern is evident from the haplotype network, but the DAPC separated both Cyprus locations from the rest of the localities (see Fig. 39). On the other hand, there are 19 haplotypes of S7 in total, and many of them are shared (10) (see Fig. 40). The result of discriminant analysis for S7 shows interconnection for all sub-populations (see Fig. 41).

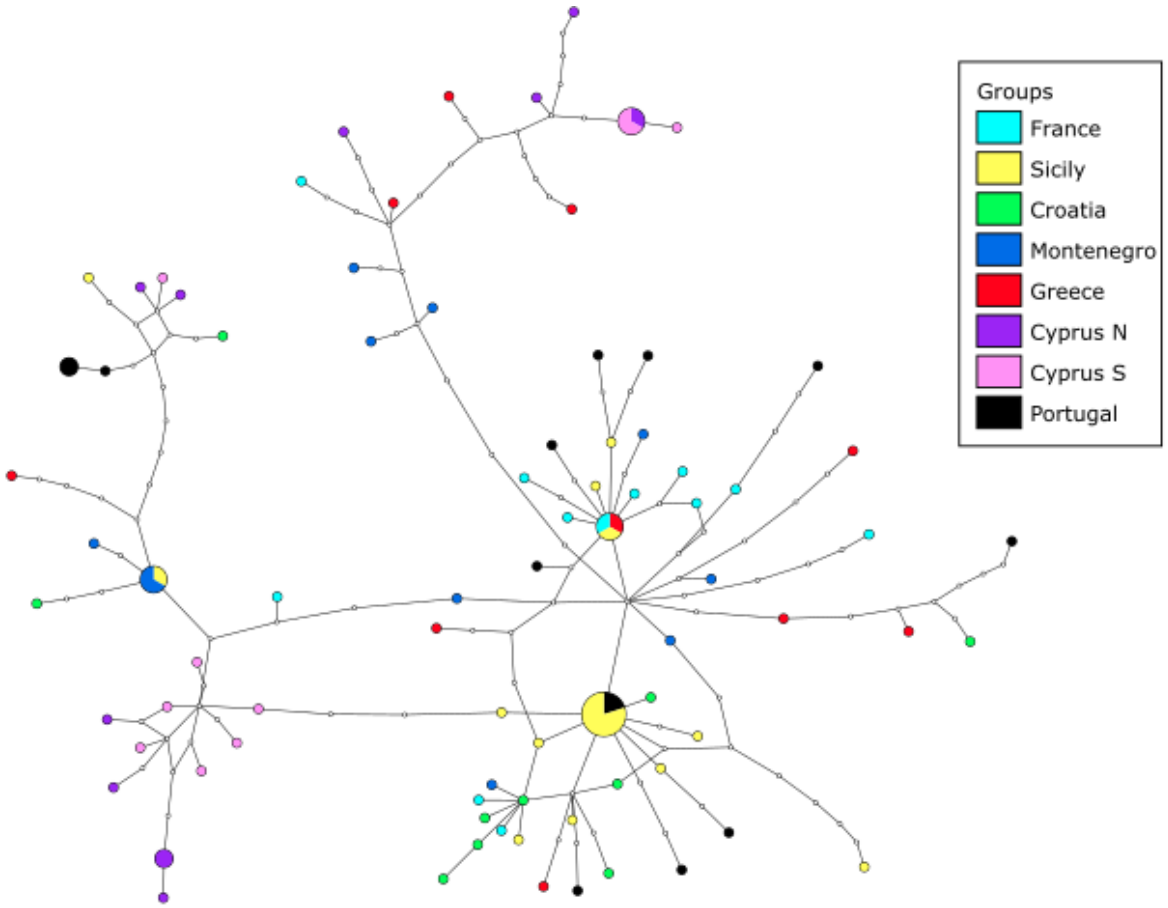

Figure 38:
Haplotype networks for *G. incognitus* for cyt b. The colours correspond to localities described in legend and area of the circles is proportional to each haplotype frequency.

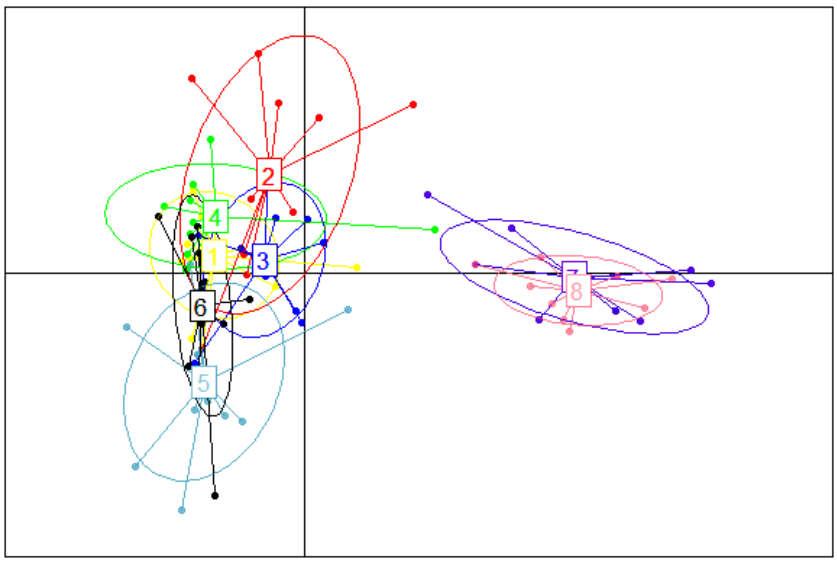

Figure 39:
Discriminant Analysis of Principal Components (DAPC) for *G. incognitus* for cyt b. Dots refer to haplotypes and inertia ellipses to groups. Colours of populations are identical to those used in the haplotype network: 1 (yellow) Sicily, 2 (red) Greece, 3 (dark blue) Montenegro, 4 (green) Croatia, 5 (light blue) France, 6 (black) Portugal, 7 (purple) Cyprus N, 8 (pink) Cyprus S.

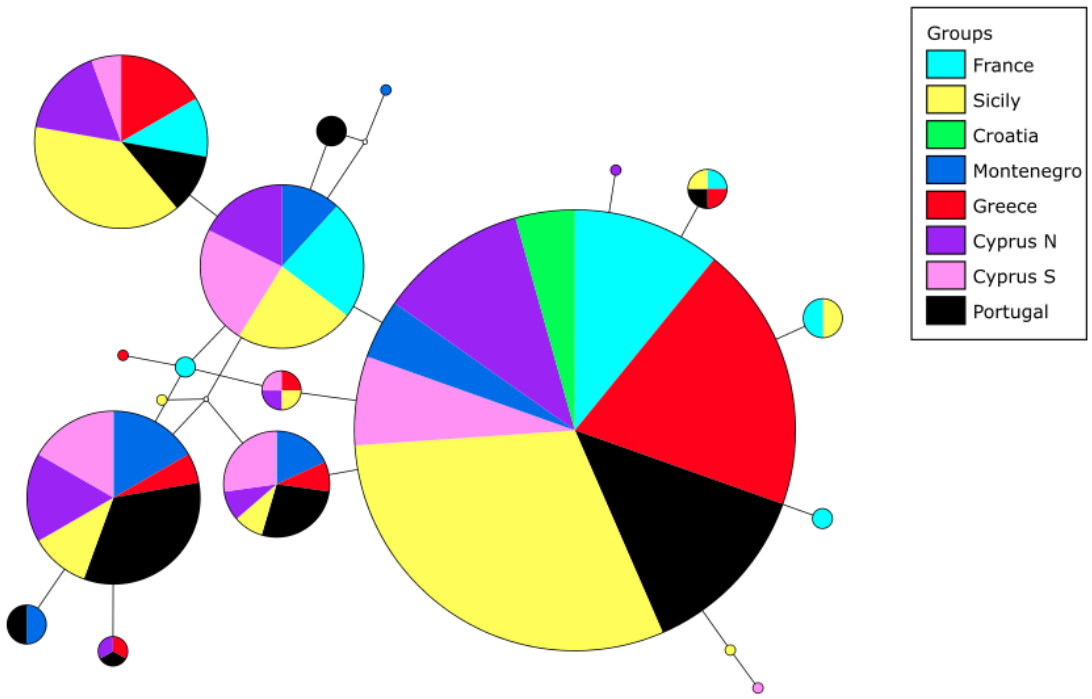

Figure 40:
Haplotype networks for *G. incognitus* for S7. The colours correspond to localities described in legend and area of the circles is proportional to each haplotype frequency.

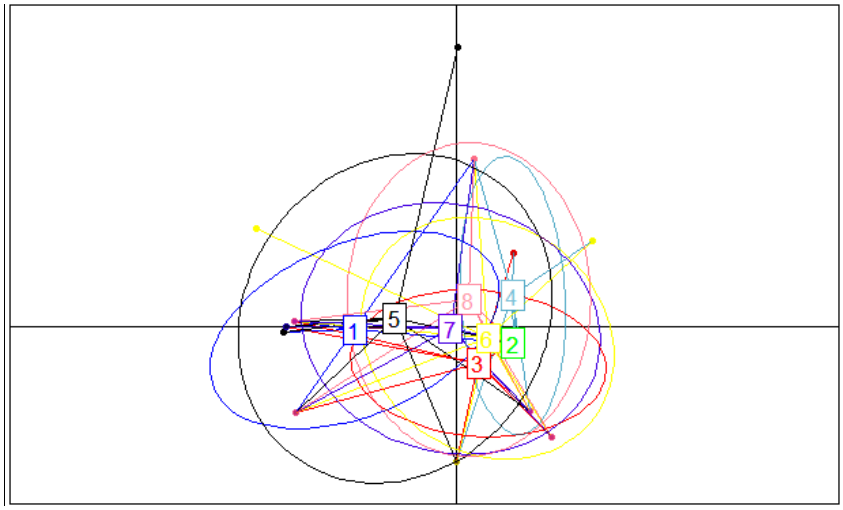

Figure 41:
Discriminant Analysis of Principal Components (DAPC) for *G. incognitus* for S7. Dots refer to haplotypes and inertia ellipses to groups. Colours of populations are identical to those used in the haplotype network: 1 (dark blue) Montenegro, 2 (green) Croatia, 3 (red) Greece, 4 (light blue) France, 5 (black) Portugal, 6 (yellow) Sicily, 7 (purple) Cyprus N, 8 (pink) Cyprus S.

The substitution model used for construction of Bayesian Skyline plot was GTR + I + G for cyt b and GTR was used for S7 as the most similar to the assessed one. Bayesian Skyline plot for cyt b shows slow population growth alternating with the rapid growth (see Fig. 42). Skyline plot for S7 shows constant population with the slight recent increase (see Fig. 44).

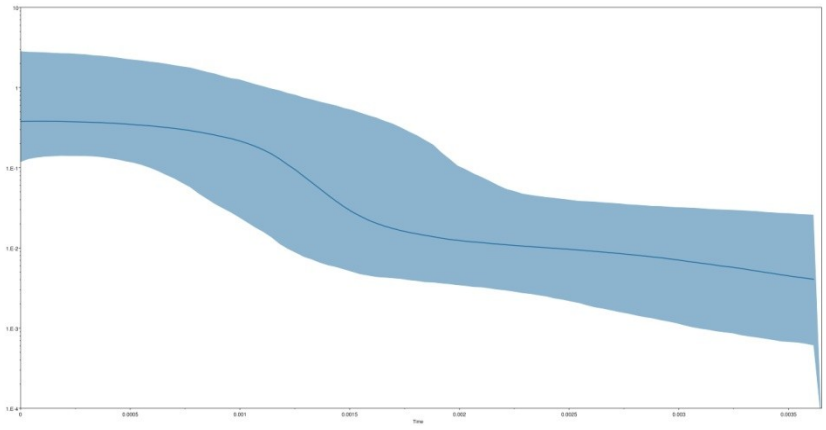

Figure 42:
Bayesian skyline plot for *G. incognitus* for cyt b. The course of the graph illustrates fluctuation of the population size from recent to the coalescence (left to the right).Y-axis stands for effective population size and x-axis for time scale in units of mutation on a nucleotide position. Blue middle line shows the median estimate and the outer lines of blue area represent 95% confidence interval.

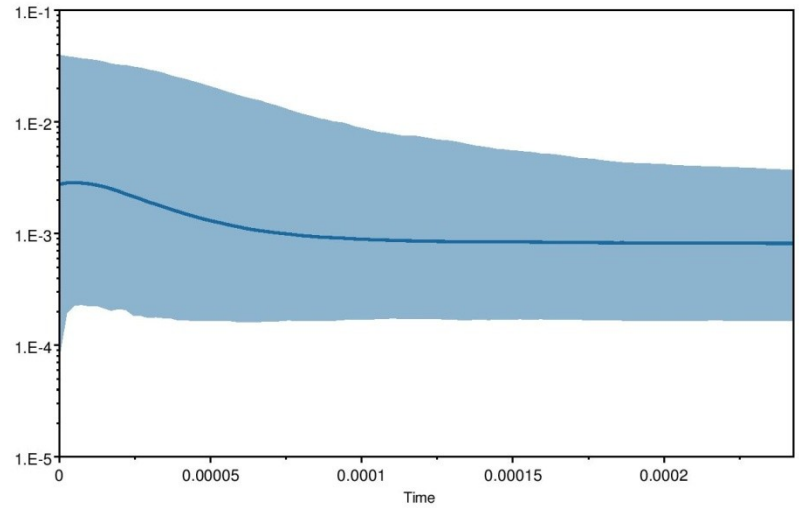

Figure 43:
Bayesian skyline plot for *G. incognitus* for S7. The course of the graph illustrates fluctuation of the population size from recent to the coalescence (left to the right).Y-axis stands for effective population size and x-axis for time scale in units of mutation on a nucleotide position. Blue middle line shows the median estimate and the outer lines of blue area represent 95% confidence interval.

Geneland divided dataset of *G. incognitus* for cyt b to four populations scattered across the range of distribution. The distribution of inferred populations can be seen in Fig 44. The result of the analysis for S7 showed that distribution of *G. incognitus* is divided into two populations. One population comprises Portugal and Montenegro and rest of the localities belong to the second population (see Fig. 45).

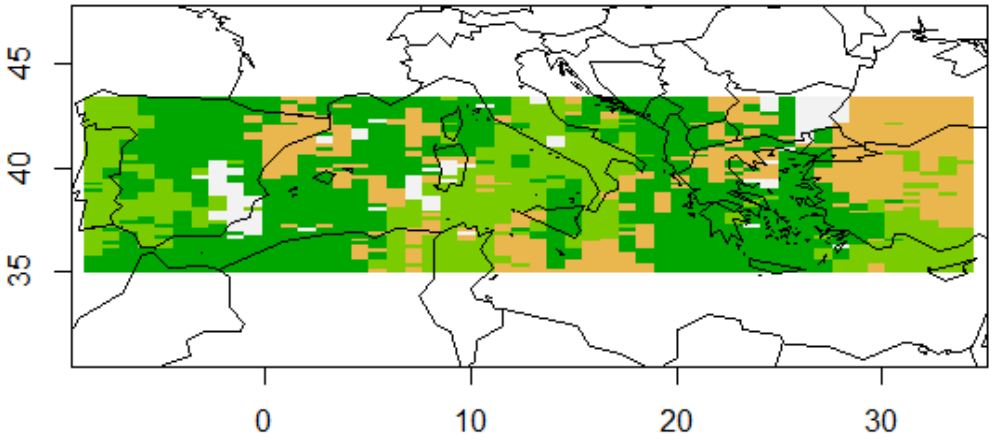

Figure 44:
Spatial distribution of populations of *G. incognitus* for cyt b by Geneland. Each colour corresponds to one sub-population. Y-axis refers to geographical latitude and x-axis geographical longitude.

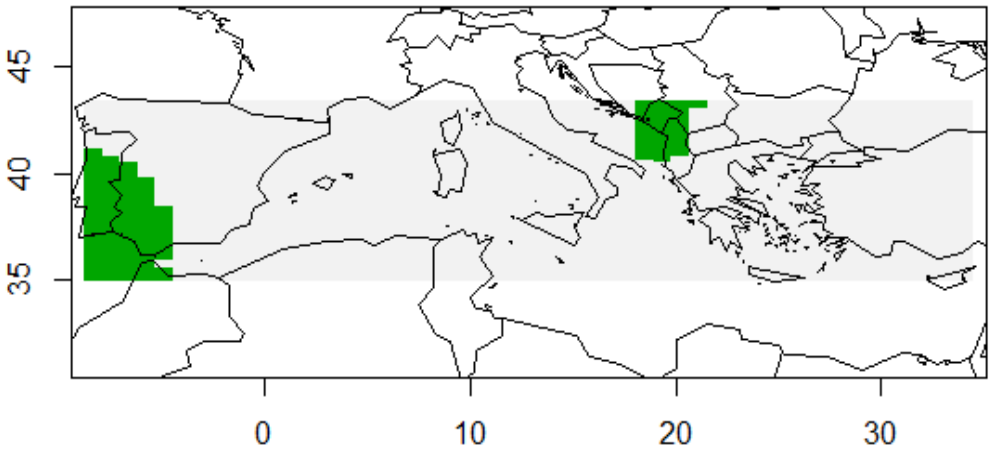

Figure 45:
Spatial distribution of populations of *G. incognitus* for S7 by Geneland. Each colour corresponds to one sub-population. Y-axis refers to geographical latitude and x-axis geographical longitude.

For cyt b, the highest uncorrected p-distances between groups (0.8 - 1.1%) are observed for subpopulations from both Cyprus localities compared with subpopulations from other localities. The lowest divergence between the groups (0.5%) is found for Sicily with Montenegro, Croatia and France. Within group distances were in the similar range of magnitude (see Tab. 30). Genetic distances for S7 are lower than for cyt b and range between 0.2 - 0.4% both between and within groups (see Fig. 31).

Table 30: Genetic distances for *G. incognitus* for cyt b (uncorrected p-distances). Db - distance between groups, Dw - distance within group.

Db	Sicily	Greece	Montenegro	Croatia	France	Portugal	Cyprus N	Cyprus S	**Dw**
Sicily									0.0042
Greece	0.007								0.0086
Montenegro	0.005	0.007							0.0057
Croatia	0.005	0.008	0.007						0.0065
France	0.005	0.007	0.006	0.007					0.0053
Portugal	0.007	0.009	0.008	0.008	0.007				0.0085
Cyprus N	0.009	0.010	0.009	0.011	0.010	0.011			0.0103
Cyprus S	0.008	0.009	0.008	0.009	0.008	0.010	0.009		0.0082

Table 31: Genetic distances for *G. incognitus* for S7 (uncorrected p- distances). Db - distance between groups, Dw - distance within group**,** n/c - not compared (only one specimen in dataset).

Db	Montenegro	Croatia	Greece	France	Portugal	Sicily	Cyprus N	Cyprus S	**Dw**
Montenegro									0.0032
Croatia	0.003								n/c
Greece	0.004	0.002							0.0029
France	0.004	0.002	0.003						0.0024
Portugal	0.003	0.003	0.004	0.004					0.0037
Sicily	0.004	0.002	0.003	0.002	0.004				0.0026
Cyprus N	0.003	0.002	0.003	0.003	0.003	0.003			0.0031
Cyprus S	0.003	0.002	0.003	0.003	0.003	0.003	0.003		0.0028

Chromogobius quadrivittatus

A total of 30 specimens were processed for mitochondrial gene cyt b. The alignment obtained for the cyt b sequences had a length of 1117 bp. The best-fit substitution model calculated by AIC and BIC was Hasegawa-Kishino-Yano (HKY). Haplotype diversity (Hd = 0.880) was high, while nucleotide diversity low (π = 0.00269) what indicates a recent expansion of population. This was also confirmed by negative values of neutrality tests (Tajima's D, Fu and Li's), which were all significant (see Tab. 32).

Table 32: Diversity measures of *C. quadrivittatus* for cyt b. Ns - number of sequences, S - number of segregating sites, Nh - number of haplotypes, Hd - haplotype diversity, π - nucleotide diversity, Fu & Li's F, Fu & Li's D and Tajima's D. Statistical significance of Fu & Li's F and D **, $p < 0.02$ and Tajima's D *, $p < 0.05$.

Species	Ns	S	Nh	Hd	π	Fu & Li's F	Fu & Li's D	Tajima's D
Chromogobius quadrivittatus	30	25	16	0.880	0.00269	**-3.89930****	**-3.99943****	**-1.86371****

In AMOVA analysis four localities (Montenegro, Croatia, Sicily, Greece) were included (see Fig. 12). Almost all genetic variability (99%) was due to variance within populations. F_{ST} index (0.00758) reached value of low differentiation. This was proven also by comparisons of pairwise F_{STs} which were generally very low and had values of 0, with the exception of Greece vs. Montenegro (0.043) and Greece vs. Croatia (0.06 – moderate differentiation), see Tab. 33 and 34.

Table 33 and 34: AMOVA and pairwise F_{STs} for *C. quadrivittatus* for cyt b. Values with statistical significance $p < 0.05$/number of pairs are shown in bold.

AMOVA	Degrees of freedom	Variance components	% of variation	F_{ST}
among populations	2	0.01145	0.76	0.00758
within populations	27	1.50000	99.24	

pairwise F_{STs}	Montenegro	Croatia	Sicily	Greece
Montenegro	0.000			
Croatia	**0.000**	0.000		
Sicily	**0.000**	**0.000**	0.000	
Greece	**0.043**	0.060	**0.000**	0.000

Haplotype network for cyt b exhibits only two shared haplotypes. One is shared between Montenegro and Croatia and the other is shared with Sicily too. The subpopulation from Greece does not share any haplotype with any other locality (see Fig. 46). Discriminant analysis did not separate any subpopulation (see Fig. 47).

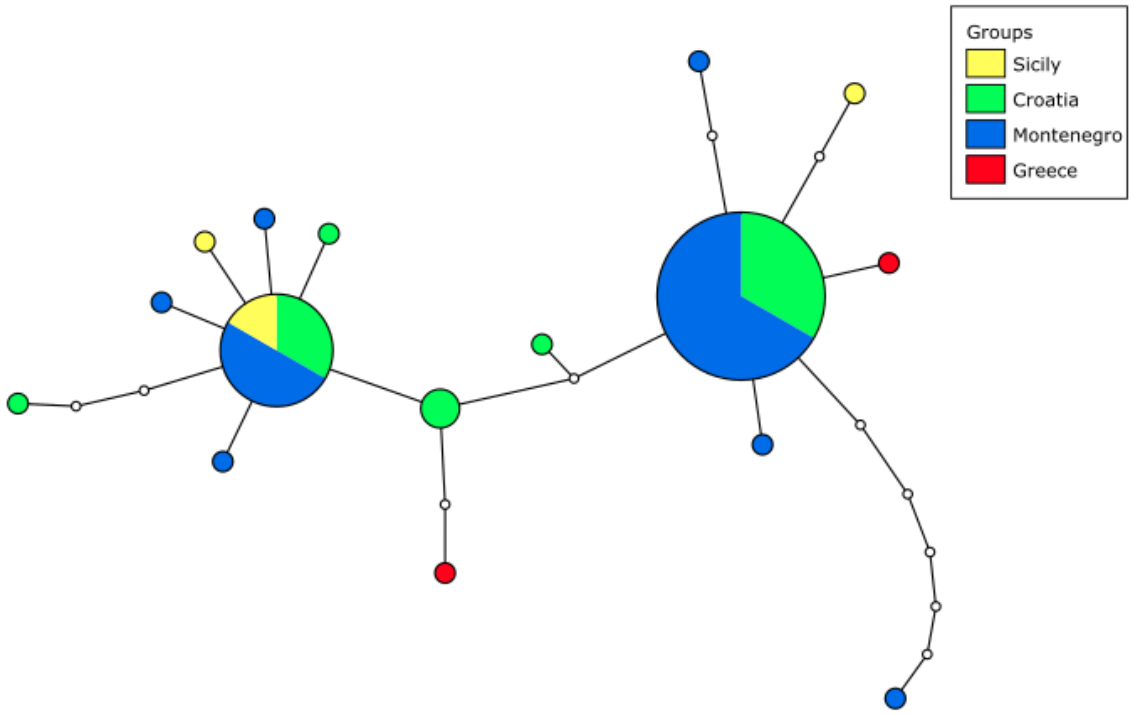

Figure 46:
Haplotype network for *C. quadrivittatus* for cyt b. The colours correspond to localities described in legend and area of the circles is proportional to each haplotype frequency.

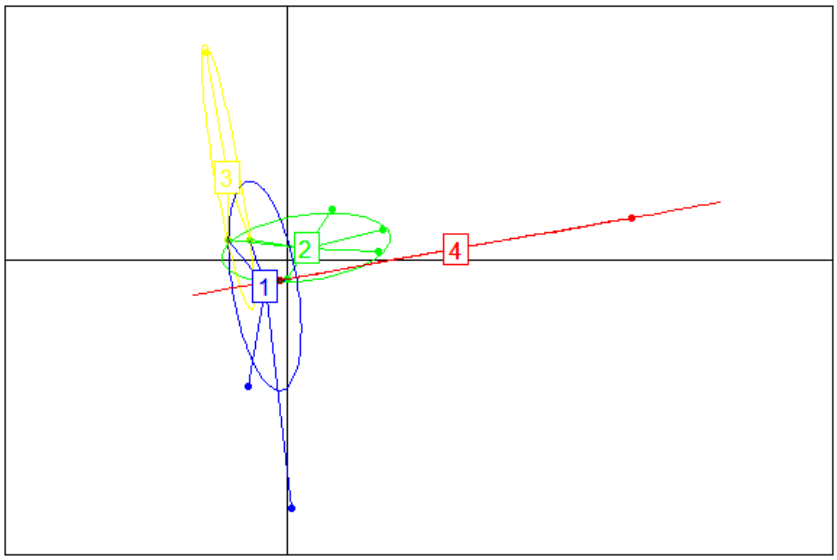

Figure 47:
Discriminant Analysis of Principal Components (DAPC) for *C. quadrivittatus* for cyt b. Dots refer to haplotypes and inertia ellipses to groups. Colours of populations are identical to those used in the haplotype network: 1 (dark blue) Montenegro, 2 (green) Croatia, 3 (yellow) Sicily, 4 (red) Greece.

The substitution model used for constructing Bayesian Skyline plot was HKY. Bayesian Skyline plot cyt b shows fast growth in the past followed by the constant population until recent (see Fig. 48).

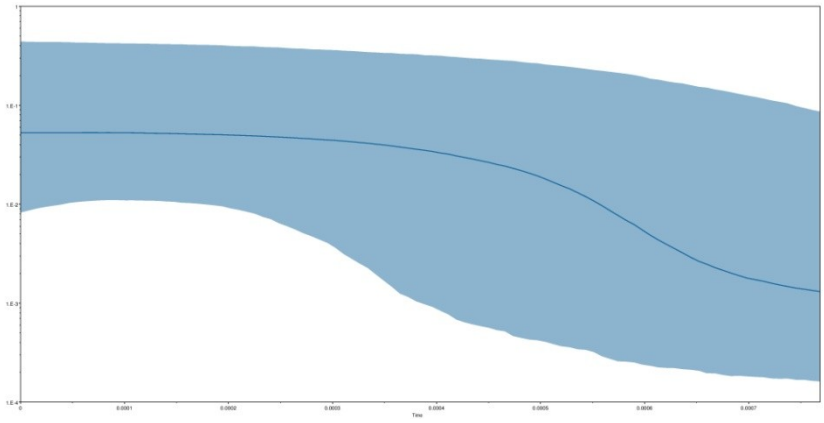

Figure 48:
Bayesian skyline plot for *C. quadrivittatus* for cyt b. The course of the graph illustrates fluctuation of the population size from recent to the coalescence (left to the right).Y-axis stands for effective population size and x-axis for time scale in units of mutation on a nucleotide position. Blue middle line shows the median estimate and the outer lines of blue area represent 95% confidence interval.

The Geneland divided *C. quadrivittatus* into two populations, one of which included Greece and Sicily and second Croatia and Montenegro. The distribution of inferred populations can be seen in Fig. 49.

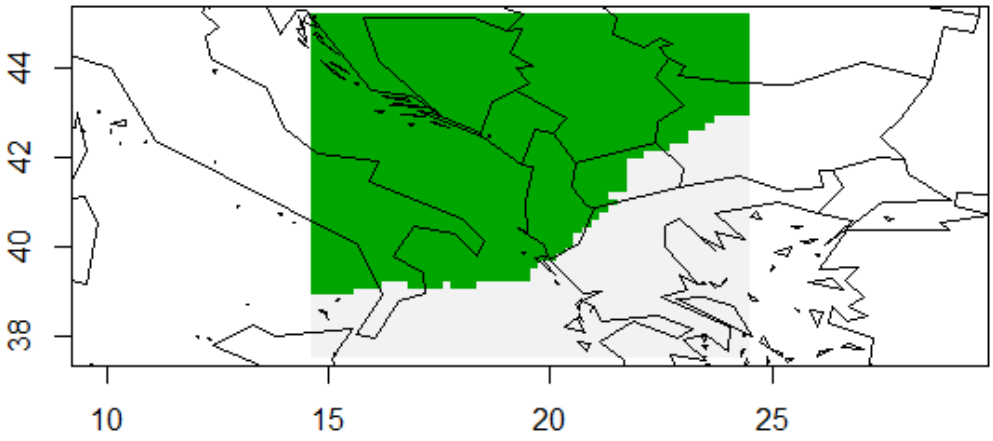

Figure 49:
Spatial distribution of populations of *C. quadrivittatus* for cyt b by Geneland. Each colour corresponds to one population. Y-axis refers to geographical latitude and x-axis geographical longitude.

Mean genetic distances (uncorrected p-distances) were calculated within and between groups, with values being similar and low. Divergence between the populations varied between 0.3 - 0.4%. The highest intraspecific distance of 0.45% appears to be in the subpopulation from Greece (see Tab. 35).

Table 35: Genetic distances for *C. quadrivittatus* for cyt b (uncorrected p-distances). Db - distance between groups, Dw - distance within group.

Db	Montenegro	Croatia	Sicily	Greece	**Dw**
Montenegro					0.0027
Croatia	0.003				0.0023
Sicily	0.003	0.003			0.0036
Greece	0.003	0.003	0.004		0.0045

Chromogobius zebratus

In total, 43 specimens assigned to *Chromogobius zebratus* based on morphology were processed and cyt b analysed. The alignments and subsequent phylogenetic analyses revealed a cryptic diversity: three lineages existing within the dataset. It turned out to be a complex of species. As the type locality of *C. zebratus* is Dalmatia in the vicinity of Split (central Adriatic Sea), we cannot assign any of the lineages to this species unless morphological examination of both the type material and our material is undertaken, as two of the revealed lineages can belong to the described species based on the distribution. Thus, 32 specimens were referred to as *Chromogobius* sp. 1, two specimens as *Chromogobius* sp. 2 and nine specimens as *Chromogobius* sp. 3. The species are sympatric in some localities; in case of the locality in Greece all three species inhabit the same site, in several localities two of the species are sympatric (see Fig. 50).

Phylogenetic trees including all species of the genus *Chromogobius* were constructed in order to explore the relationships between the discovered lineages and other species of the genus. Both Bayesian analyses and Maximum Likelihood performed on all species of the genus *Chromogobius* supported well all three lineages within *C. zebratus* complex and showed that they are closely related to each other. Bayesian analysis suggested *Chromogobius* sp. 3 as a sister group to *Chromogobius* sp. 2 and 1 (see Fig. 51); whereas Maximum Likelihood revealed all three *Chromogobius* sp. groups in unresolved position (see Fig. 52). In both analyses *C. britoi* was revealed as sister group to *C. zebratus* complex, and *C. quadrivittatus* sister group to the all other species of the genus. Interspecific genetic distances are high, 12 - 20% divergence between the three species of *Chromogobius zebratus* complex, and 26-38% between one of the species of the complex and two remaining species of the genus, *C. quadrivittatus* and *C. britoi*. The intraspecific distances were very low, the highest was 0.8%, revealed within the group *Chromogobius* sp. 1 (see Tab. 36).

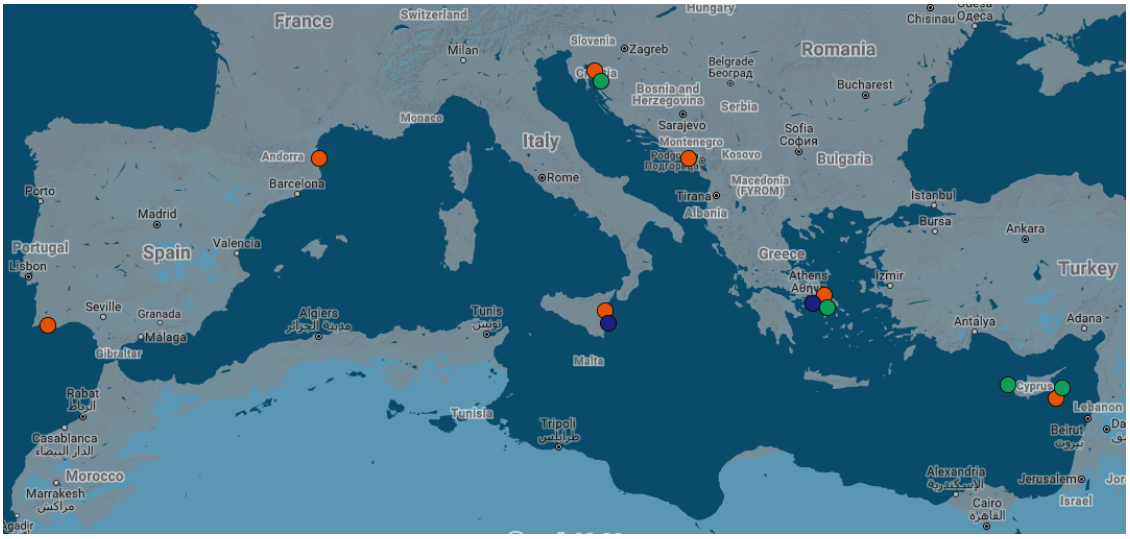

Figure 50: Map of sampling sites of the complex *Chromogobius zebratus*. Orange colour corresponds to *Chromogobius* sp. 1, blue to *Chromogobius* sp. 2 and green to *Chromogobius* sp. 3.

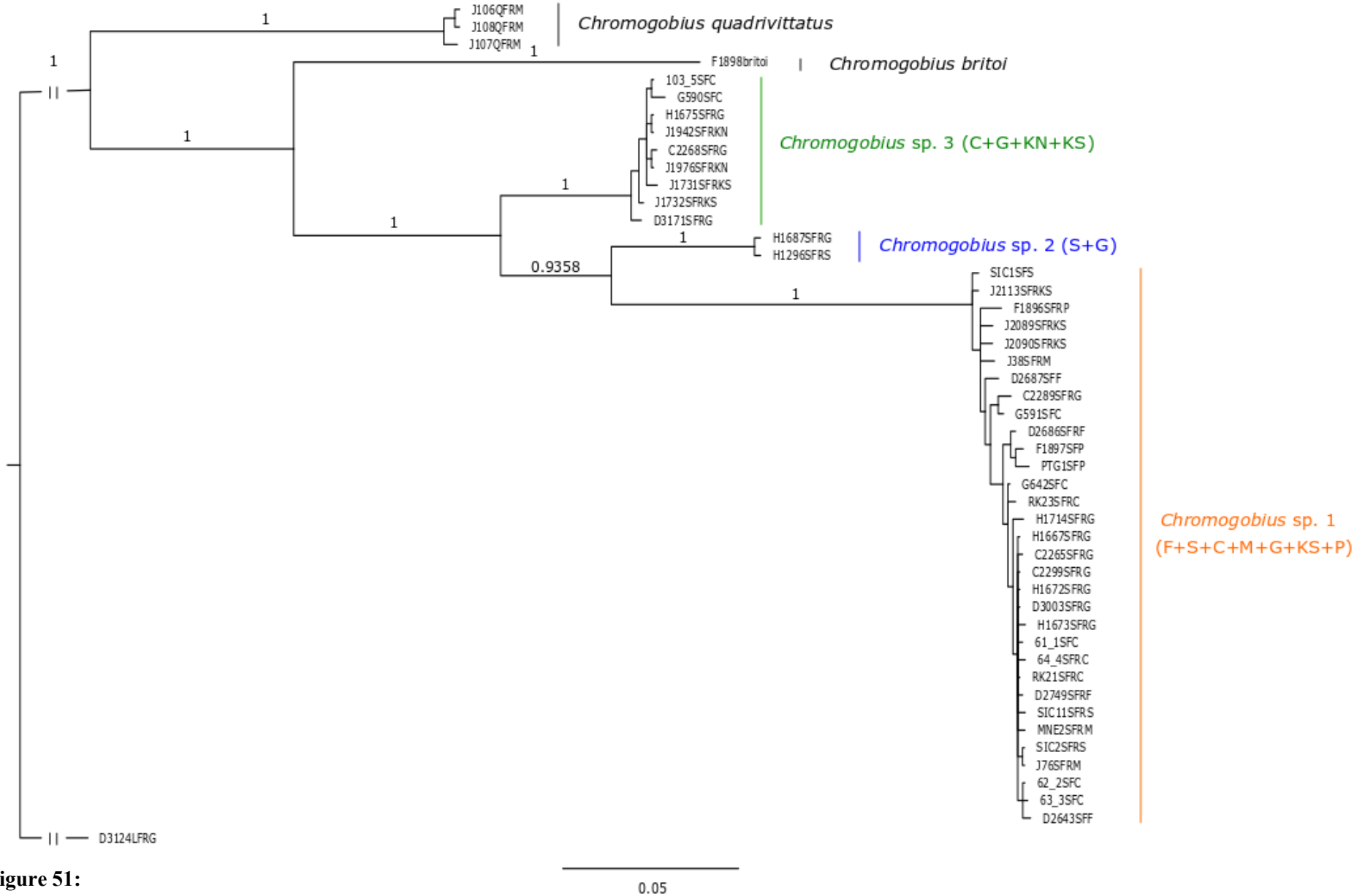

Figure 51:
Phylogenetic tree for cyt b constructed by the Bayesian inference. The numbers on the branches represent posterior probabilities. Only values higher than 0.90 have been displayed. Last letter in sample code and letters in brackets respond to localities (F- France, S- Sicily, C- Croatia, M- Montenegro, G- Greece, KN- Cyprus north, KS- Cyprus south, and P- Portugal).

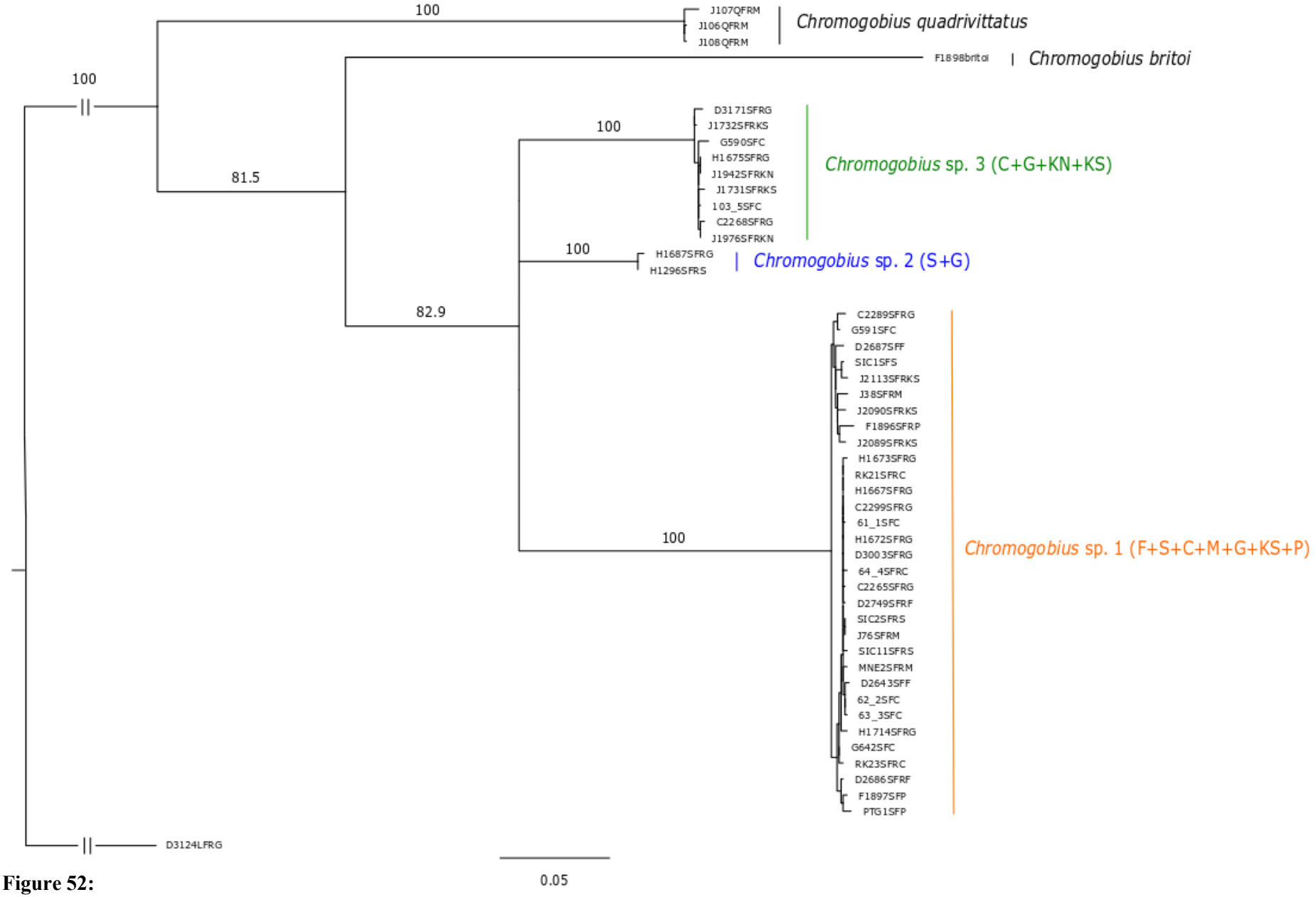

Figure 52:
Phylogenetic tree for cyt b constructed by the Maximum Likelihood. The numbers on the branches represent bootstrap values, in %. Only values higher than 75% have been displayed. Last letter in the sample code and letters in brackets respond to localities (F- France, S- Sicily, C- Croatia, M- Montenegro, G- Greece, KN- Cyprus north, KS- Cyprus south, and P- Portugal).

Table 36: Genetic distances for cyt b for the genus *Chromogobius* (uncorrected p-distances). Db - distance between species, Dw - distance within species, n/c - not compared (only one specimen in dataset).

Db		1	2	3	4	5	Dw
1	*Chromogobius* sp. 1						0.0075
2	*Chromogobius* sp. 2	0.177					0.0027
3	*Chromogobius* sp. 3	0.202	0.123				0.0045
4	*C. quadrivittatus*	0.327	0.314	0.315			0.0054
5	*C. britoi*	0.345	0.286	0.259	0.375		n/c

Due to a small number of individuals in other groups of *C. zebratus* complex, subsequent analyzes were carried out only for the group *Chromogobius* sp. 1 with the exception of haplotype network where all three groups were depicted.

Haplotype network was constructed for the whole *Chromogobius zebratus* complex for cyt b. There was a clear separation of the three networks. *Chromogobius* sp. 3 exhibits only one shared haplotype, shared between Cyprus N and Greece. *Chromogobius* sp. 1 has two shared haplotypes; one bigger haplotype is shared between several specimens from Greece and few Croatian specimens. Besides that there is a prevalence of unique haplotypes (see Fig. 53).

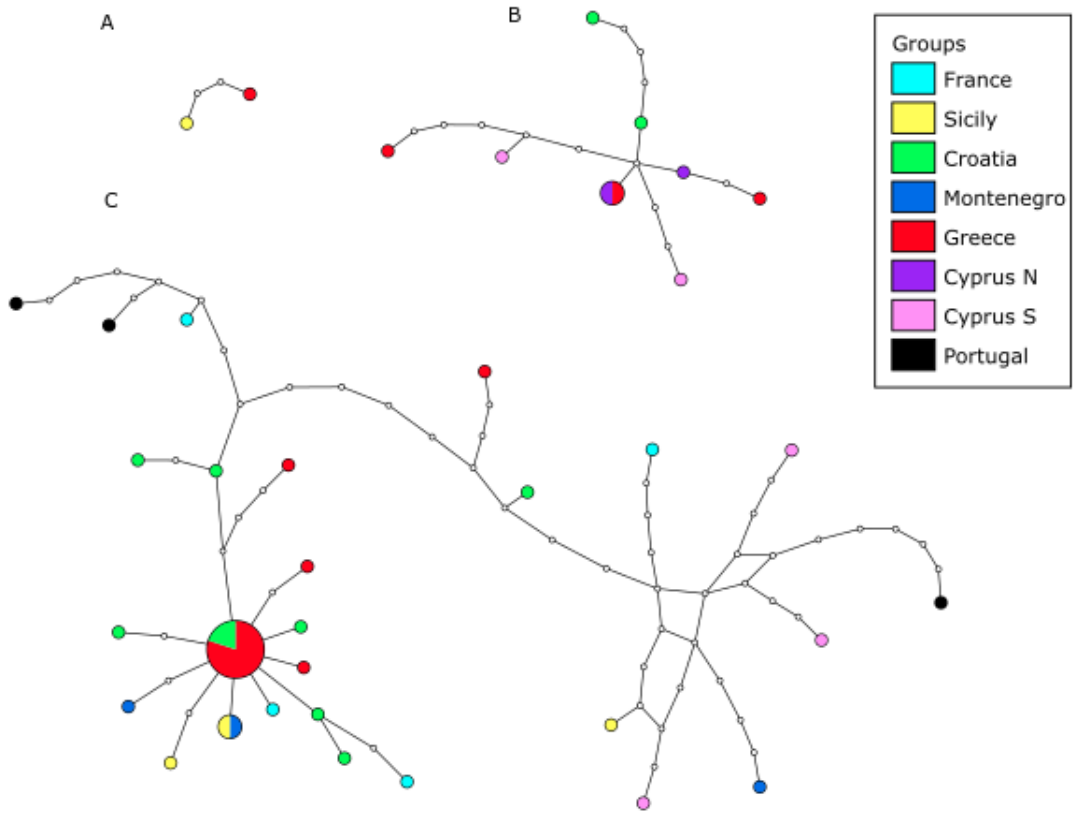

Figure 53:
Haplotype networks for cyt b (A - *Chromogobius* sp. 2, B - *Chromogobius* sp. 3, C - *Chromogobius* sp. 1. The colours correspond to localities described in legend and area of the circles is proportional to each haplotype frequency.

A total of 32 specimens of *Chromogobius* sp. 1 were processed for analyses of the mitochondrial gene cyt b. The alignment had a length of 1117 bp. The best-fit substitution model calculated by AIC and BIC was General Time Reversible with invariant sites (GTR+I). Haplotype diversity (Hd = 0.978) was high, while nucleotide diversity low (π = 0.00741) what indicates recent population expansion. This was also confirmed by negative values of neutrality tests (Tajima's D, Fu and Li's), which were all significant (see Tab. 37).

Table 37: Diversity measures of *Chromogobius* sp. 1 for cyt b. Ns - number of sequences, S - number of segregating sites, Nh - number of haplotypes, Hd - haplotype diversity, π - nucleotide diversity, Fu & Li's F, Fu & Li's D and Tajima's D. Statistical significance of Fu & Li's F and D and Tajima's D *, $p < 0.05$.

Species	Ns	S	Nh	Hd	π	Fu & Li's F	Fu & Li's D	Tajima's D
Chromogobius zebratus sp. 1	32	62	27	0.978	0.00741	**-2.97602***	**-2.86741***	**-1.81620***

In AMOVA analysis seven localities (Greece, Croatia, Montenegro, France, Portugal, Sicily, Cyprus south (S)) were included (see Fig. 49). Most of the genetic variability (82%) was due to variance within populations. F_{ST} index (0.17656) was significant and reached value of high differentiation. Comparisons of pairwise F_{STs} between seven localities had values from 0 to 0.571 (Cyprus S with Greece). The most differentiated were Portugal and Cyprus when compared with other localities, but values were not significant (see Tab. 38 and 39).

Table 38 and 39: AMOVA and pairwise F_{STs} for *Chromogobius* sp. 1 for cyt b.
Values with statistical significance $p < 0.05$/number of pairs are shown in bold.

AMOVA	Degrees of freedom	Variance components	% of variation	F_{ST}
among populations	5	0.75760	17.66	**0.17656**
within populations	26	3.53322	82.34	

pairwise F_{STs}	Greece	Croatia	Montenegro	France	Portugal	Sicily	Cyprus S
Greece	0.000						
Croatia	**0.000**	0.000					
Montenegro	**0.024**	**0.000**	0.000				
France	**0.024**	**0.000**	**0.000**	0.000			
Portugal	0.341	0.289	**0.043**	**0.016**	0.000		
Sicily	**0.000**	**0.001**	**0.000**	**0.000**	0.095	0.000	
Cyprus S	0.571	0.522	0.236	0.295	0.198	0.271	0.000

Discriminant analysis separated Portugal from all and Cyprus from almost all other localities (see Fig. 54).

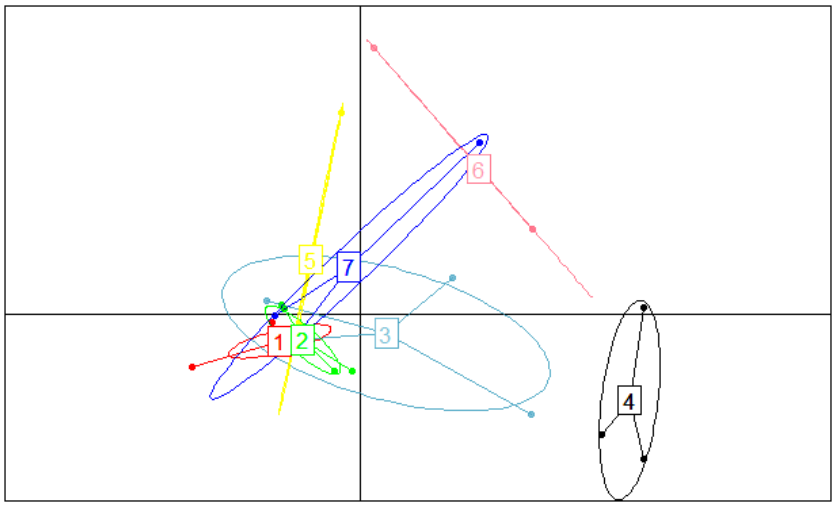

Figure 54:
Discriminant Analysis of Principal Components (DAPC) for *Chromogobius* sp. 1 for cyt b. Dots refer to haplotypes and inertia ellipses to groups. Colours of populations are identical to those used in the haplotype network: 1 (red) Greece, 2 (green) Croatia, 3 (light blue) France, 4 (black) Montenegro, 5 (yellow) Sicily, 6 (pink) Cyprus S, 7 (dark blue) Montenegro.

The substitution model used to construct Bayesian Skyline plot was HKY. Bayesian Skyline plot for cyt b shows slow growth in the past with stabilization in recent (see Fig. 55).

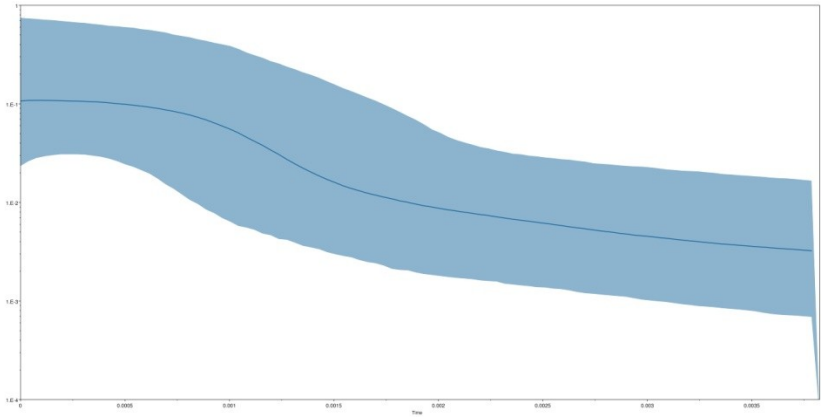

Figure 55:
Bayesian skyline plot for *Chromogobius* sp. 1 for cyt b. The course of the graph illustrates fluctuation of the population size from recent to the coalescence (left to the right).Y-axis stands for effective population size and x-axis for time scale in units of mutation on a nucleotide position. Blue middle line shows the median estimate and the outer lines of blue area represent 95% confidence interval.

The Geneland divided *Chromogobius* sp. 1 into three populations. One of them has a disjunctive areal, which combines France and Cyprus. The second population combines samples from Greece, Croatia, Montenegro and Sicily, and the third from Portugal and Sicily. The distribution of inferred populations can be seen in Fig 56.

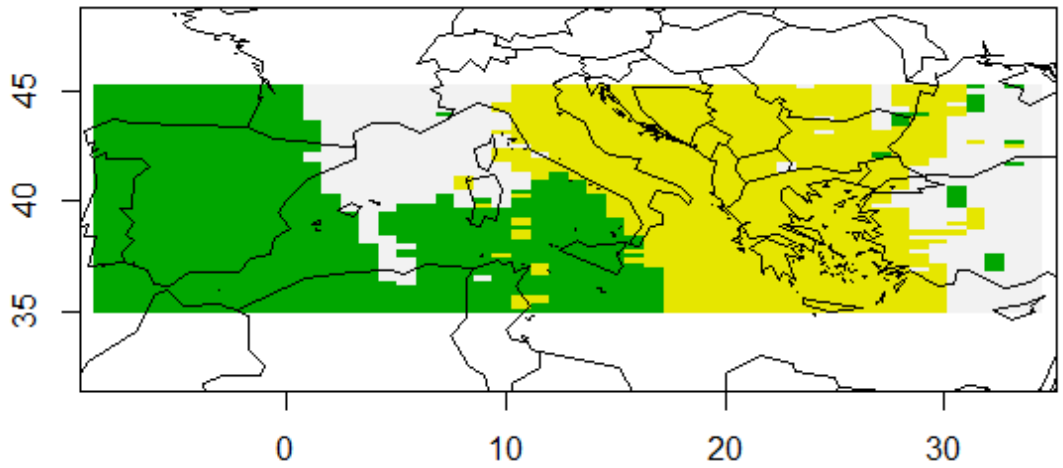

Figure 56:
Spatial distribution of populations of *Chromogobius* sp. 1for cyt b by Geneland. Each colour corresponds to one population.Y-axis refers to geographical latitude and x-axis geographical longitude.

Mean genetic distances (uncorrected p-distances) were computed within and between groups. Genetic distances between groups vary between 0.4 - 1.3%. The results confirm the highest divergence of 1.3% between Portugal and Cyprus as they are the most distant locations from each other. The highest within group distance was for Portugal with a value of 1.3% (see Tab. 40), where one haplotype differs significantly from the remaining two (see Fig. 53).

Table 40: Genetic distances for *Chromogobius* sp. 1 for cyt b (uncorrected p-distances). Db - distance between groups, Dw - distance within group.

Db	Greece	Croatia	France	Portugal	Sicily	Cyprus S	Montenegro	**Dw**
Greece								0.0040
Croatia	0.004							0.0045
France	0.006	0.006						0.0085
Portugal	0.011	0.011	0.011					0.0134
Sicily	0.005	0.006	0.007	0.012				0.0079
Cyprus S	0.012	0.012	0.012	0.013	0.011			0.0078
Montenegro	0.006	0.006	0.008	0.012	0.007	0.011		0.0091

Complex Zebrus - Millerigobius

Two closely related species were analysed, *Zebrus zebrus* and *Millerigobius macrocephalus*. In total, 19 specimens assigned to *Millerigobius macrocephalus* and 18 specimens assigned to *Zebrus zebrus* based on morphology were processed and cyt b analysed. The alignments and subsequent phylogenetic analyses revealed a cryptic diversity: a third lineage existing within the dataset. Of 19 specimens previously assigned to *M. macrocephalus*, 13 were assigned to *Millerigobius macrocephalus* and six specimens to *Zebrus* cf. *zebrus* based on cyt b analysis. The species occur in sympatry in some localities; in the case of Montenegro all three of them, inhabit the same area (see Fig.57). Phylogenetic trees including complex *Zebrus* - *Millerigobius* were constructed in order to explore the relationships between the detected lineages. Bayesian analyses and Maximum Likelihood both supported well all lineages and revealed *Zebrus* cf. *zebrus* to be a sister group to *Zebrus zebrus*. Moreover, analyses revealed one considerably divergent specimen in two groups. Sample MillerMC in the lineage *M. macrocephalus* and sample H1723 in the lineage *Zebrus* cf. *zebrus* (see Fig. 58 and 59). Therefore, mean genetic distances (uncorrected p- distances) were calculated in two ways, 1) with these two abovementioned individuals (MillerMC and H1723) as separate lineages (see Tab. 41) and 2) with these individuals included in the most closely related lineage to them (see Tab. 42). The interspecific distance between *Zebrus zebrus* and *Zebrus* cf. *zebrus* was 25 and 24.8%, respectively, based on two ways of calculation. The p-distance between either of these two species and *Millerigobius* was in the range 23.1 to 23.9%. The sample MillerMC was distant by 3.7% from *M. macrocephalus* and the sample H1723 by 5.3 % from *Zebrus* cf. *zebrus*. Intraspecific distance was the highest in *Zebrus zebrus* (0.8%). Including H1723 and MillerMC specimens in lineages created higher distances within groups with the highest value of 2% in *Zebrus* cf. *zebrus*.

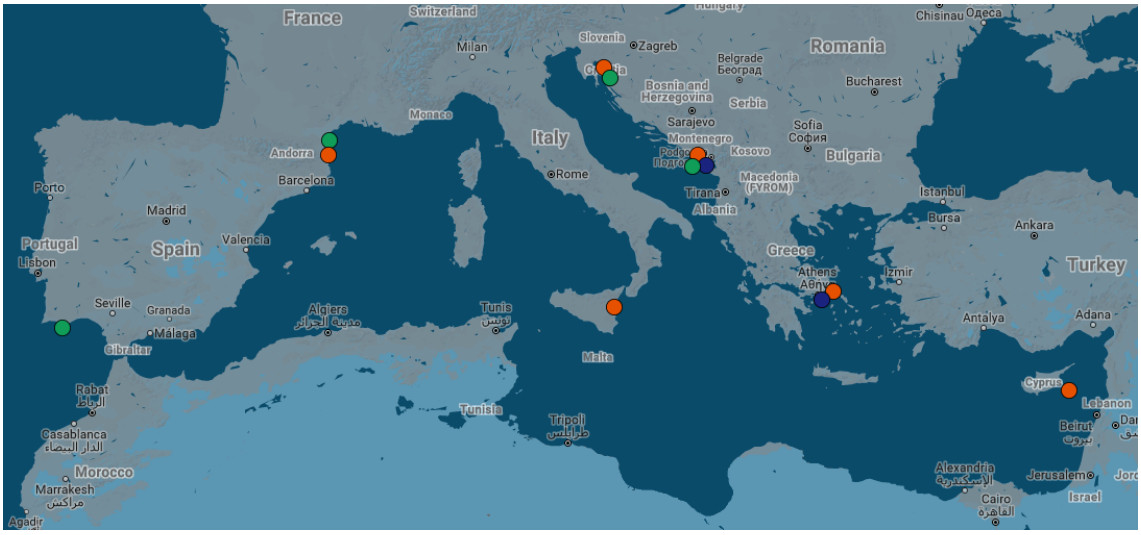

Figure 57: Map of sampling sites of complex *Zebrus* - *Millerigobius*. Orange colour corresponds to *Millerigobius macrocephalus*, blue to *Zebrus* cf. *zebrus* and green to *Zebrus zebrus*.

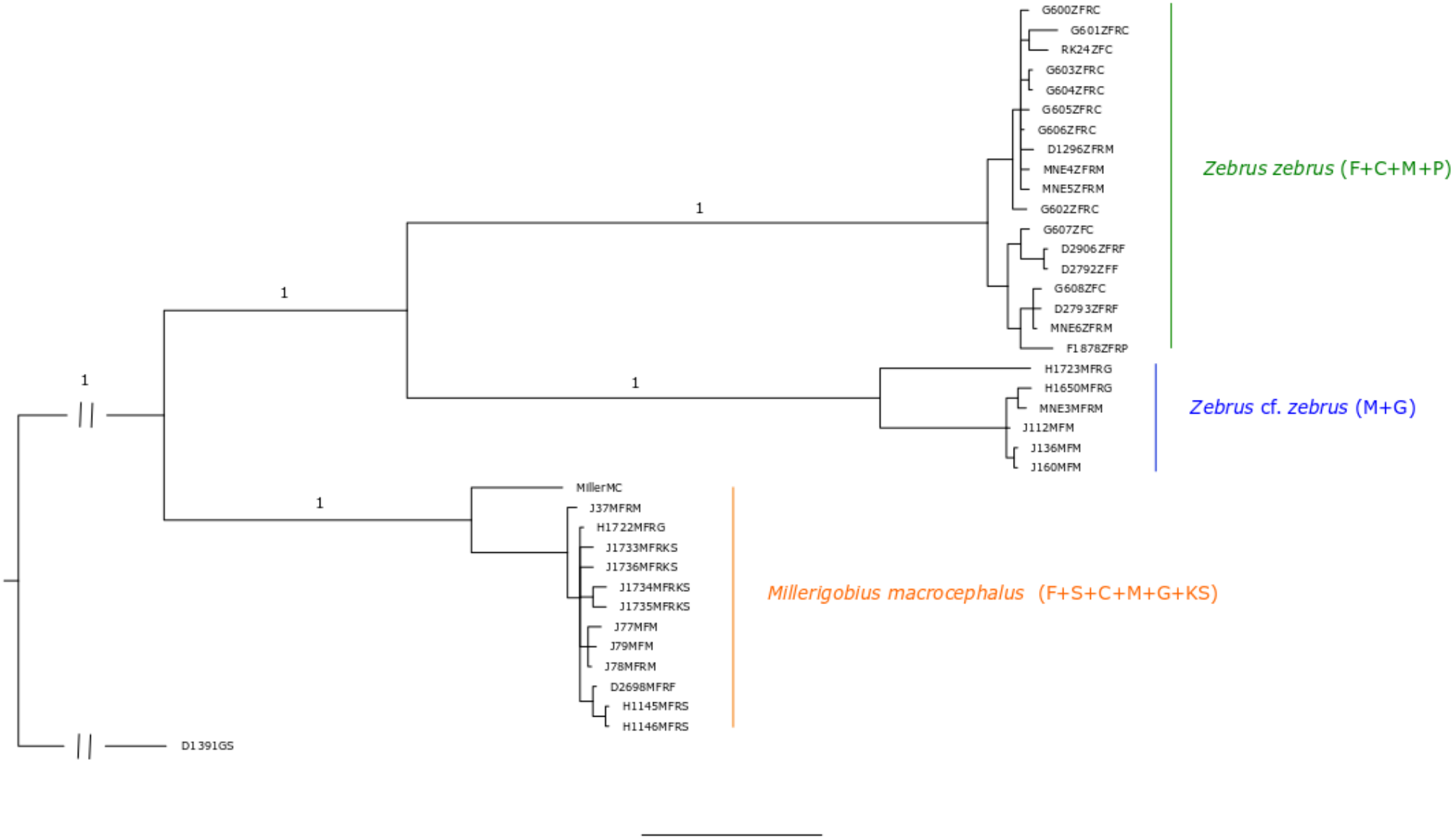

Figure 58:
Phylogenetic tree for *Millerigobius – Zebrus* complex for cyt b constructed by the Bayesian inference. The numbers on the branches represent posterior probabilities. Only values higher than 0.90 have been displayed. Last letter in the sample code and letters in brackets correspond to localities (F- France, S- Sicily, C- Croatia, M- Montenegro, G- Greece, KS- Cyprus south, and P- Portugal).

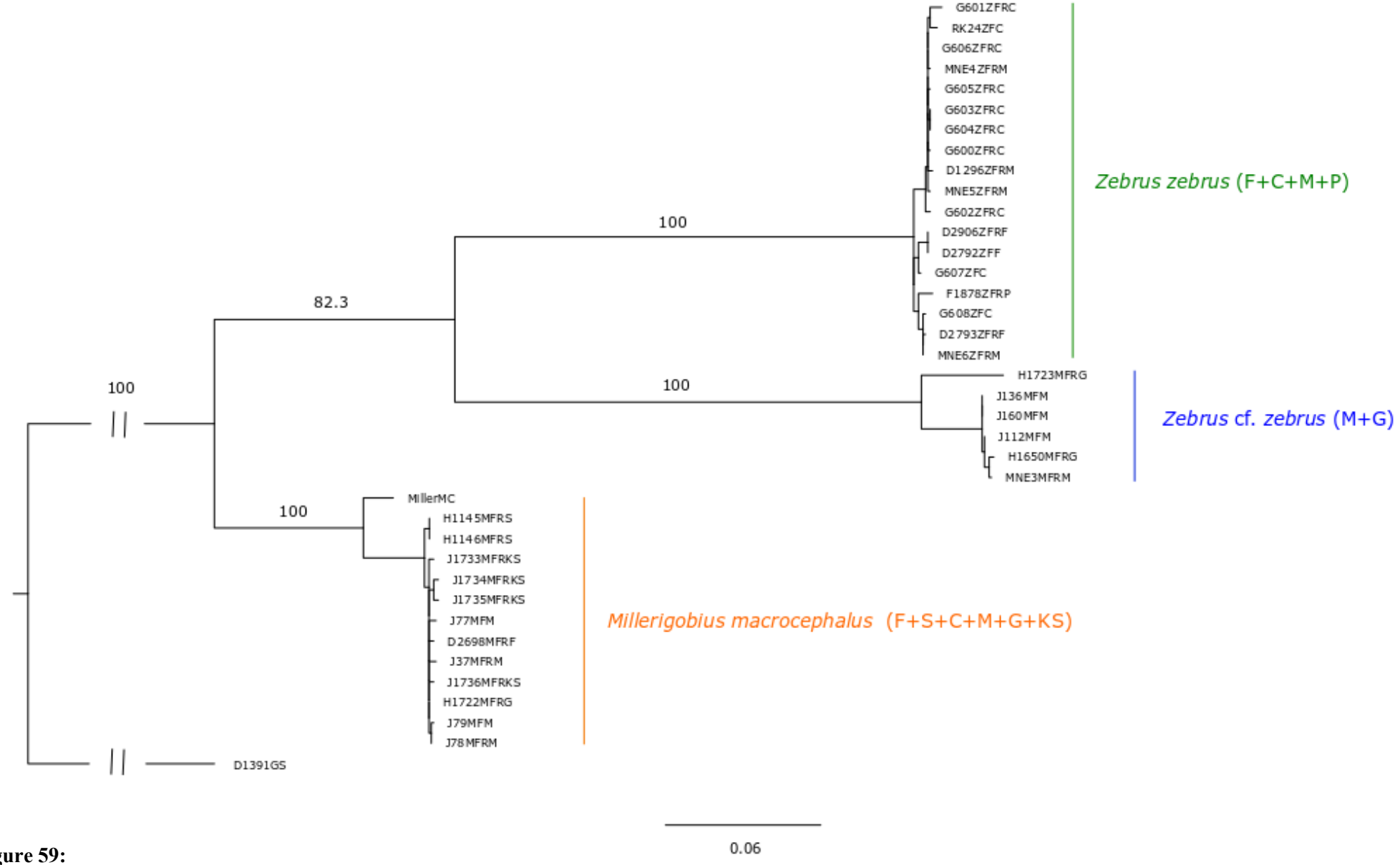

Figure 59:

Phylogenetic tree for *Millerigobius – Zebrus* complex for cyt b constructed by the Maximum Likelihood. The numbers on the branches represent bootstrap values, in %. Only values higher than 75% have been displayed. Last letter in the sample code and letters in brackets respond to localities (F- France, S- Sicily, C- Croatia, M- Montenegro, G- Greece, KS- Cyprus south, and P- Portugal).

Table 41 and 42: Genetic distances for *Millerigobius – Zebrus* complex for cyt b computed for all separate groups and for three lineages below. Db - distance between groups, Dw - distance within group, n/c - not compared (only one specimen in dataset).

Db	**1**	**2**	**3**	**4**	**5**	**Dw**
1 *Millerigobius macrocephalus*						0.0043
2 MillerMC	0.037					n/c
3 H1723	0.239	0.243				n/c
4 *Zebrus* cf. *zebrus*	0.230	0.229	0.053			0.0027
5 *Zebrus zebrus*	0.236	0.228	0.242	0.250		0.0081

Db	**1**	**2**	**3**	**Dw**
1 *Millerigobius macrocephalus* + MillerMC				0.0093
2 *Zebrus* cf. *zebrus* + H1723	0.231			0.0194
3 *Zebrus zebrus*	0.235	0.248		0.0081

Haplotype network was constructed for all three lineages for cyt b with a clear separation of these networks. In this case, I used connection limit of 60 steps in order to depict the number of mutations separating the individuals H1723 and MillerMC from the rest of "their lineages". Using 95% connection limit, these individuals would be shown as separated. Generally, there are only a few haplotypes shared by more individuals (and are shared by two specimens only), most haplotypes are unique, see Fig. 60.

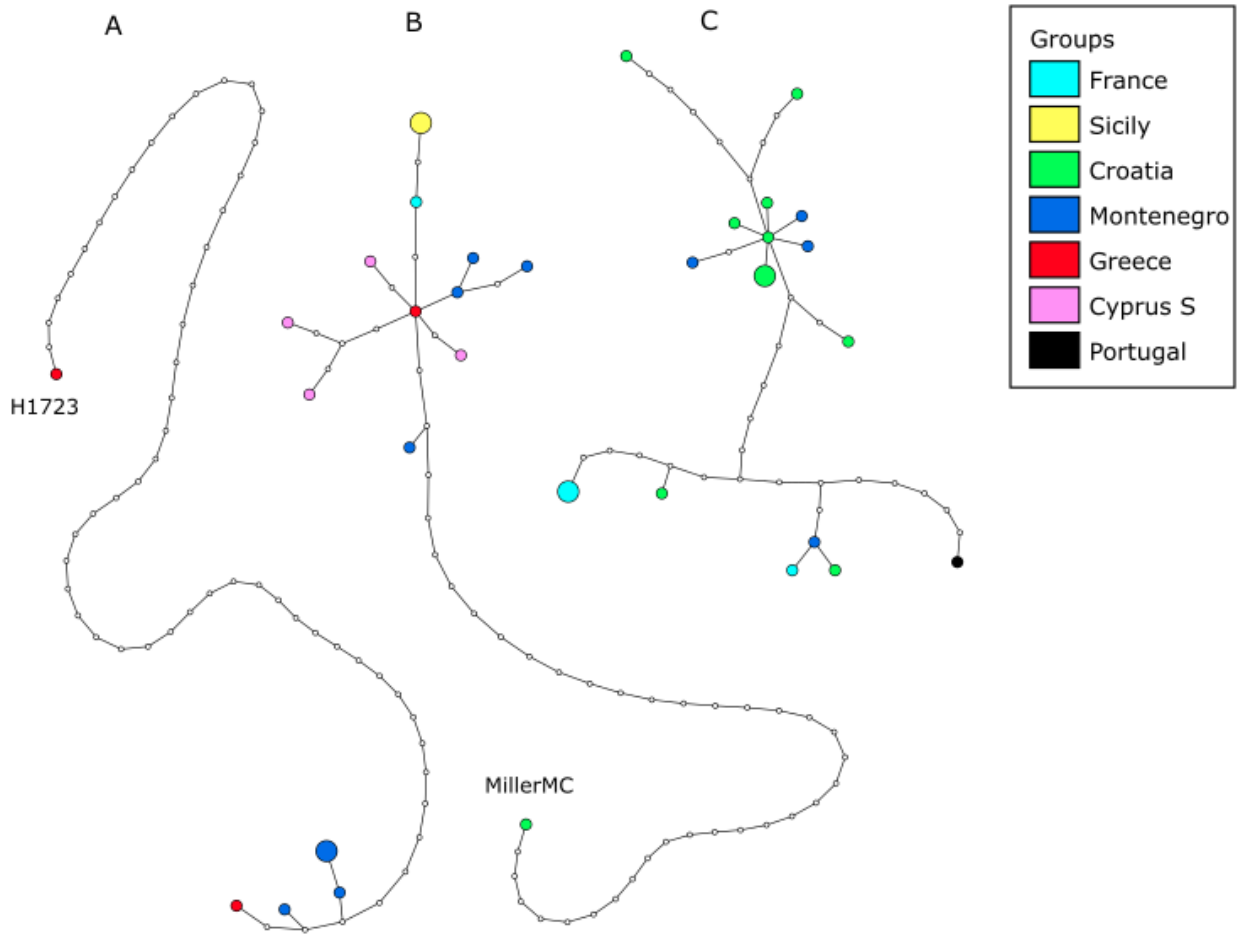

Figure 60:
Haplotype networks for cyt b (A - *Zebrus* cf. *zebrus*, B - *Millerigobius macrocephalus*,C - *Zebrus zebrus*. The colours correspond to localities described in legend and area of the circles is proportional to each haplotype frequency.

For the analyses of the genetic structure, the original dataset was split into three lineages with small number of individuals in each of them. Subsequent analyzes were carried out for the *Millerigobius macrocephalus* and *Zebrus zebrus*, but the numbers of analysed individuals were very low and the results should be considered orientational.

Millerigobius macrocephalus

A total of 13 specimens were processed for mitochondrial gene cyt b. The alignment obtained for the cyt b sequences had a length of 1115 bp. The best-fit substitution model calculated by AIC and BIC was General Time Reversible (GTR) but for AIC with gamma distribution (GTR+G). Haplotype diversity (Hd = 0.987) was high, while nucleotide diversity low (π = 0.00904) what indicates recent population expansion. This was also confirmed by negative values of neutrality tests, where Fu & Li's D and Tajima's D were significant (see Tab. 43).

Table 43: Diversity measures of *M. macrocephalus* for cyt b. Ns - number of sequences, S - number of segregating sites, Nh - number of haplotypes, Hd - haplotype diversity, π - nucleotide diversity, Fu & Li's F, Fu & Li's D and Tajima's D. Statistical significance of Fu & Li's F and Tajima's D *, $p < 0.05$.

Species	Ns	S	Nh	Hd	π	Fu & Li's F	Fu & Li's D	Tajima's D
Millerigobius macrocephalus	13	54	12	0.987	0.00904	**-2.42442***	-2.20192	**-1.88980***

In AMOVA analysis six localities (Greece, Montenegro, Croatia, France, Sicily, Cyprus south (S)) were included (see Fig. 56). According to the results there is absence of genetic structure among the populations. All genetic variation is explained by the variability within the populations. Zero F_{ST} index indicates panmictic population and thus no differentiation occurs. Comparisons of pairwise F_{STs} between six localities had values from 0 to quite high value 1.0 which was reached in six cases (see Tab. 44 and 45).

Table 44 and 45: AMOVA and pairwise F_{STs} for *M. macrocephalus* for cyt b.
Values with statistical significance $p < 0.05$/number of pairs are shown in bold.

AMOVA	Degrees of freedom	Variance components	% of variation	F_{ST}
among populations	4	-0.52623	-10.69	0
within populations	8	5.45000	110.69	

pairwise F_{STs}	Greece	Montenegro	Croatia	France	Sicily	Cyprus S
Greece	0.000					
Montenegro	**0.000**	0.000				
Croatia	1.000	0.911	0.000			
France	1.000	0.176	1.000	0.000		
Sicily	1.000	0.601	1.000	1.000	0.000	
Cyprus S	**0.000**	0.159	0.868	**0.000**	0.467	0.000

DAPC analysis interconnected Greece, Cyprus and Montenegro and rest of localities were distant including clearly divergent Croatian sample MillerMC (see Fig. 61).

Figure 61:
Discriminant Analysis of Principal Components (DAPC) for *M. macrocephalus* for cyt b. Dots refer to haplotypes and inertia ellipses to groups. No ellipse means one specimen at the given locality. Colours of populations are identical to those used in the haplotype network: 1 (dark blue) Montenegro, 2 (red) Greece, 3 (yellow) Sicily, 4 (light blue) France, 5 (pink) Cyprus S, 6 (green) Croatia.

The substitution model used to construct the Bayesian Skyline plot applied was GTR. The Bayesian Skyline plot for cyt b shows constant population size with a moderate decline and subsequent increase and again slight decline in present (see Fig. 62).

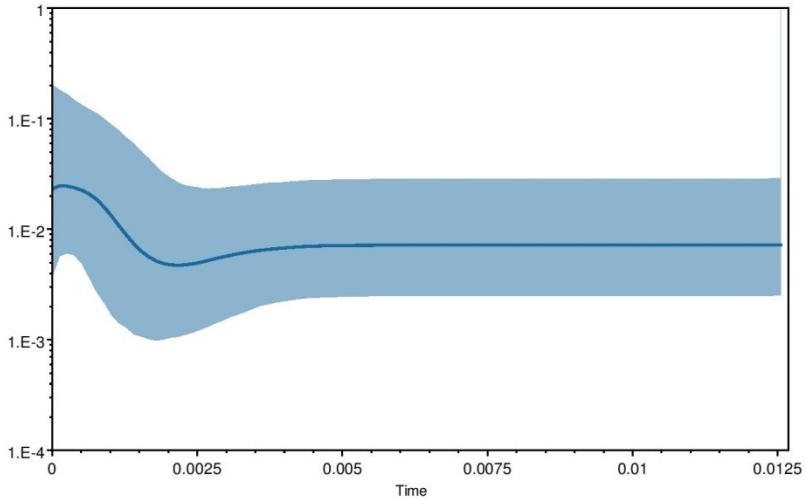

Figure 62:
Bayesian skyline plot for *M. macrocephalus* for cyt b. The course of the graph illustrates fluctuation of the population size from recent to the coalescence (left to the right).Y-axis stands for effective population size and x-axis for time scale in units of mutation on a nucleotide position. Blue middle line shows the median estimate and the outer lines of blue area represent 95% confidence interval.

The result of analysis by Geneland shows that *M. macrocephalus* is divided into two populations, but this is due to a very big distance of the Croatian sample MillerMC. This analysis included Croatia and Sicily in one population, which does not correlate with a haplotype network because of the highest number of mutations between these sites. The rest of the localities were grouped in one population. The distribution of inferred populations can be seen in Fig. 63.

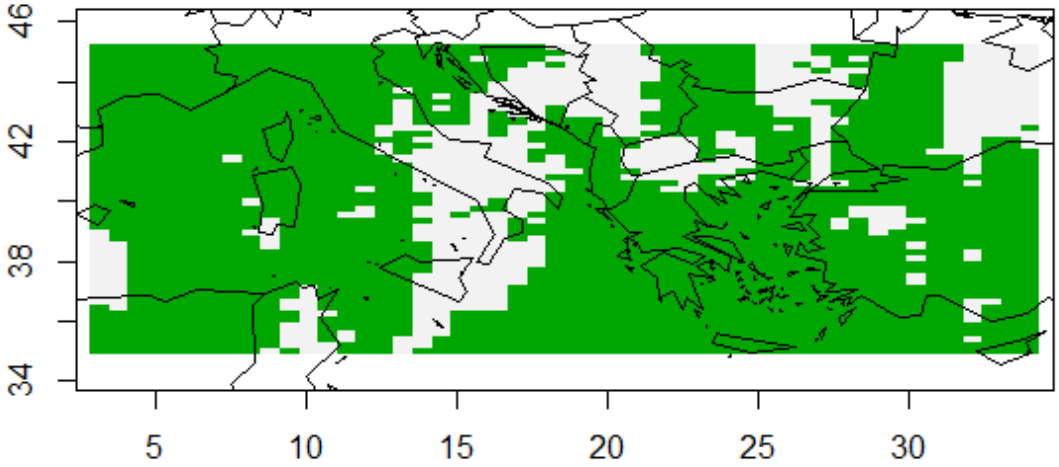

Figure 63:
Spatial distribution of populations of *M. macrocephalus* for cyt b by Geneland. Each colour corresponds to one population. Y-axis refers to geographical latitude and x-axis geographical longitude.

Mean genetic distances (uncorrected p-distances) were computed within and between groups. Clearly highest between-group distances were for Croatia with 3.6 - 3.8% divergence. The rest of the localities showed 0.2 - 0.6% divergences. Intraspecific distances were performed in only half of the sites because of the lack of individuals in the populations, but in the remaining distances were very low (see Tab. 46).

Table 46: Genetic distances for *M. macrocephalus* for cyt b (uncorrected p-distances). Db - distance between groups, Dw - distance within group, n/c - not compared (only one specimen in dataset).

Db	Greece	Montenegro	France	Sicily	Cyprus S	Croatia	**Dw**
Greece							n/c
Montenegro	0.002						0.0032
France	0.002	0.004					n/c
Sicily	0.004	0.006	0.002				0
Cyprus S	0.003	0.005	0.005	0.006			0.0048
Croatia	0.036	0.037	0.036	0.036	0.038		n/c

Zebrus zebrus

A total of 18 sequences of specimens of 1117 bp length were processed for mitochondrial gene cyt b. The best-fit substitution model calculated by AIC and BIC was General Time Reversible with invariant sites (GTR+I). Haplotype diversity (Hd = 0.987) was greater than nucleotide diversity (π = 0.00797) what indicates recent population expansion. This was also confirmed by negative values of neutrality tests (Tajima's D, Fu and Li's), none of which were significant, however (see Tab. 47).

Table 47: Diversity measures of *Z. zebrus* for cyt b. Ns - number of sequences, S - number of segregating sites, Nh - number of haplotypes, Hd - haplotype diversity, π - nucleotide diversity, Fu & Li‘s F, Fu & Li’s D and Tajima’s D.

Species	Ns	S	Nh	Hd	π	Fu & Li's F	Fu & Li's D	Tajima's D
Zebrus zebrus	18	39	16	0.987	0.00797	-1.09772	-0.97482	-0.87563

In AMOVA analysis four localities (Croatia, Montenegro, France, Portugal) were included (see Fig. 18). Genetic variation was explained almost the same by variability among populations (45%) and within populations (55%). F_{ST} index (0.45125) was significant and reached value of very high differentiation. Comparisons of pairwise F_{STs} between four localities had values from 0 (Montenegro with Croatia), the closest locations, to 0.530 (Portugal with Croatia). The highest pairwise differences were clearly for Portugal, which is the only location from the Atlantic Ocean in the dataset (see Tab. 48 and 49)

Table 48 and 49: AMOVA and pairwise F_{STs} for *Z. zebrus* for cyt b.
Values with statistical significance $p < 0.05$/number of pairs are shown in bold.

AMOVA	Degrees of freedom	Variance components	% of variation	F_{ST}
among populations	2	2.77893	45.13	**0.45125**
within populations	15	3.37937	54.87	

pairwise F_{STs}	Croatia	Montenegro	France	Portugal
Croatia	0.000			
Montenegro	**0.000**	0.000		
France	0.425	0.360	0.000	
Portugal	0.530	0.462	0.333	0.000

The result of DAPC confirms high distance of the subpopulation from Portugal from the subpopulations from the other localities and shows the interconnection of subpopulations from Montenegro with Croatia (see Fig. 64).

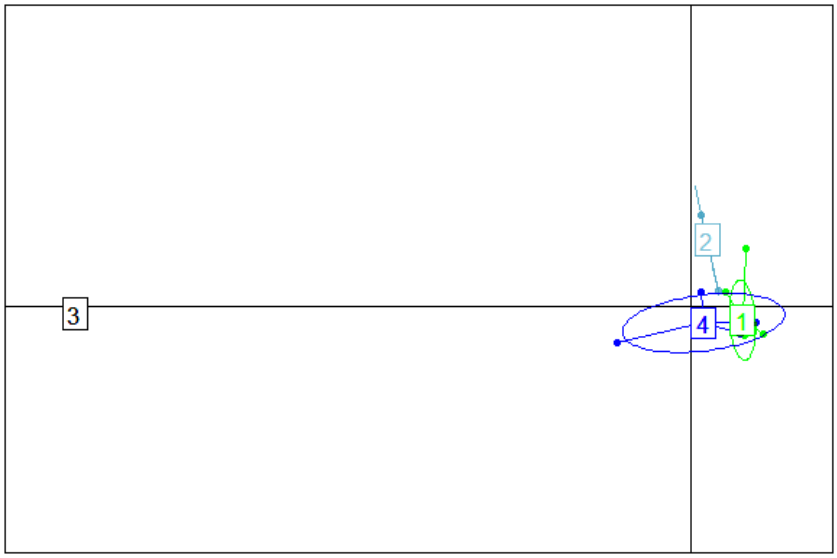

Figure 64:
Discriminant Analysis of Principal Components (DAPC) for *Z. zebrus* for cyt b. Dots refer to haplotypes and inertia ellipses to groups. No ellipse means one specimen at the given locality. Colours of populations are identical to those used in the haplotype network: 1 (green) Croatia, 2 (light blue) France, 3 (black) Portugal, 4 (dark blue) Montenegro.

Graphical record of population dynamics was estimated by method of Bayesian Skyline plot. The substitution model used to construct Bayesian Skyline plot was GTR. The Bayesian Skyline plot for cyt b shows relatively constant population with a slight growth in the more recent past (see Fig. 65).

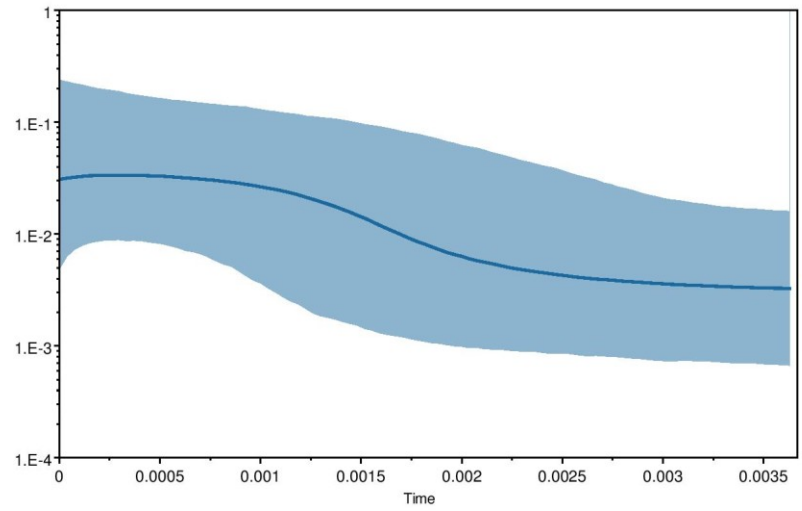

Figure 65:
Bayesian skyline plot for *Z. zebrus* for cyt b. The course of the graph illustrates fluctuation of the population size from recent to the coalescence (left to the right).Y-axis stands for effective population size and x-axis for time scale in units of mutation on a nucleotide position. Blue middle line shows the median estimate and the outer lines of blue area represent 95% confidence interval.

The Geneland divided four localities of *Z. zebrus* into three populations, where only Montenegro and Croatia are grouped together and create one population. The distribution of inferred populations can be seen in Fig. 66.

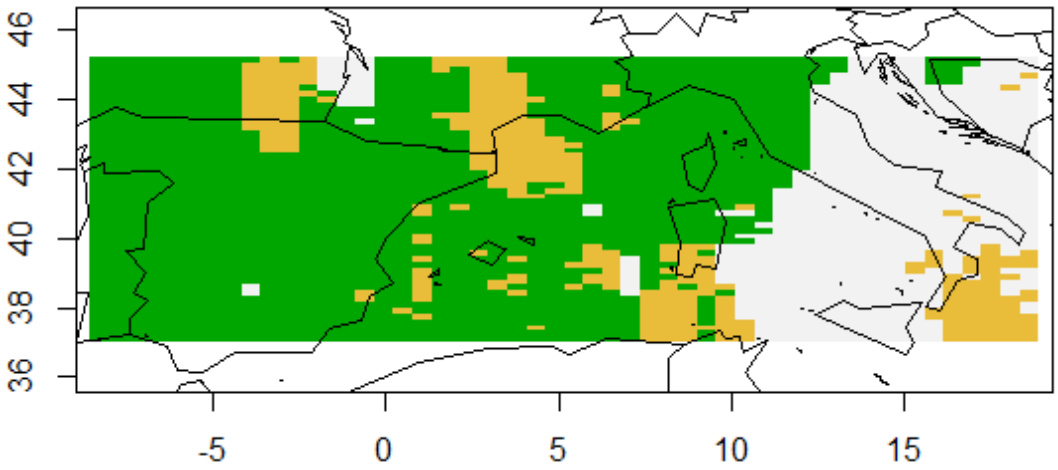

Figure 66:
Spatial distribution of populations of *Z. zebrus* by Geneland. Each colour corresponds to one population. Y-axis refers to geographical latitude and x-axis to geographical longitude.

Mean genetic distances (uncorrected p-distances) were computed within and between groups. The results confirm the highest distance 1.3% between samples from Portugal and Croatia and the lowest distance, 0.6%, between samples from Montenegro and Croatia. The greatest distance within groups was in the case of France, 0.7% (see Tab. 50).

Table 50: Genetic distances for *Z. zebrus* for cyt b (uncorrected p-distances). Db - distance between groups, Dw - distance within group, n/c - not compared (only one specimen in dataset).

Db	Croatia	France	Portugal	Montenegro	**Dw**
Croatia					0.0063
France	0.011				0.0067
Portugal	0.013	0.010			n/c
Montenegro	0.006	0.010	0.012		0.0064

Discussion

Many previous phylogeographic studies have confirmed geographical structure in populations of animals within north-eastern Atlantic and Mediterranean region, e.g. in crustaceans (Luttikhuizen et al., 2008; Palero et al., 2008; Zitari-Chatti et al., 2009) and fishes (Bahri-Sfar et al., 2000; Debes et al., 2008; Gonçalo, 2014; Innocentiis et al., 2004; Pappalardo et al., 2017; Viñas et al., 2004; Zardoya et al., 2004). However, European marine gobies have been little studied so far. Most of the papers were focused mainly on representatives of the genus *Pomatoschistus*, which often inhabits lagoons and shallow coastal waters with fine substrates and can therefore be easily caught by commercial fishing methods. Studies of *P. minutus* showed both a significant difference in population from the north of the Adriatic Sea from other populations under study, and the existence of smaller differences between the west-Mediterranean and Atlantic populations (Boissin et al., 2011; Gysels et al., 2004b; Stefanni and Thorley, 2003; Stefanni et al., 2003). Small differences between western Mediterranean and Atlantic populations were also detected in *P. microps* (Gysels et al., 2004a; Tougard et al., 2014). Within the Mediterranean region, genetic differences between different geographically distant populations of *P. marmoratus* and *P. tortonesei* were observed (Mejri et al., 2009, 2011). One research was done in *Aphia minuta* and *Gobius niger,* two ecologically divergent species (Giovannotti et al., 2009). There were found differences in *Gobius niger* between geographically distant populations (Giovannotti et al., 2009), whereas in pelagic species *Aphia minuta* no differences between populations from the same areas as *G. niger,* were observed. There are no other phylogeographic studies on the gobies of European seas.

My work has dealt with the identification of possible geographical structure and the comparison of genetic diversity in geographically distant populations of eight species of European gobies belonging to the *Gobius*-lineage, Gobiidae, Gobiiformes *(C. liechtensteini, G. cruentatus, G. geniporus, G. incognitus*, *C. quadrivittatus, C. zebratus, M. macrocephalus* and *Z. zebrus*). The results of cytochrome b analyses show a certain gradient, i.e. a difference in genetic diversity between the localities on the easternmost and westernmost end of the distribution of most of the studied species rather than some population subdivision. A certain degree of genetic partitioning and potential existence of geographical barriers was suggested in two species, *G. geniporus* and *G. cruentatus,* both rather large epibenthic species. This was confirmed by F_{ST} indices which showed a high degree of differentiation in both species.
In the case of *G. cruentatus*, there was a spatially organized structure revealed between the western and eastern part of the studied area. Haplotype network (Fig. 28) represented two groups of haplotypes, where two Atlantic localities (Portugal and Spain) together with France are obviously distinct from those from Cyprus. The same was confirmed by DAPC (Fig. 29). This was also shown by Geneland analyses, where the Atlantic and western Mediterranean formed one population and the rest of the Mediterranean Sea the second (Fig. 32). These results indicate a presence of a barrier that prevents the passage of the population from the

west of the Mediterranean to the east and vice versa. But, we have to take into account that the subpopulations of Croatia, Montenegro and Sicily in the central part of the Mediterranean have higher affinity to eastern populations (according to Geneland results), and belong to both haplogroups according to the haplotype network (Fig. 28). The potential role in this case could play the unidirectional water circulation in the Sicily Channel which was also evidenced as a breakpoint for gene flow in other marine fishes, e.g. European anchovies, *Engraulis encrasicolus* (Bembo et al., 1996), sea bass, *Dicentrarchus labrax* (Bahri-Sfar et al., 2000), European sprat, *Sprattus sprattus* (Debes et al., 2008), and *Pomatoschistus tortonesei* (Mejri et al., 2009). As there are two distinct haplogroups in this species (eastern and western), but haplotypes from the localities in the central Mediterranean Sea belong to both of them, one of the possible explanation might be that in the past two populations existed and came to a contact in the central part of the Mediterranean Sea. However, more dense sampling could reveal a completely different picture, so these results should be taken cautiously and as provisional. This species is quite rare, although considered widespread in Mediterranean Sea and North-Eastern Atlantic, it can be found in low abundances. Moreover, at different localities, we observed it at quite different depths (e.g. in Croatia 5-10 m, while in Cyprus at the depths bellow 30 m).

Similarly, the results of *G. geniporus* suggested a presence of barriers, which could prevent migration, but their definition is not easy. The distribution inferred by Geneland (Fig. 37) for cyt b divided individuals into three distinct sub-regions: western (France and Sicily), eastern (Cyprus and Greece) and northern central Mediterranean (Croatia and Montenegro), similar separation was revealed by DAPC (Fig. 34). Despite the fact that the samples from the individual sub-regions were not exclusively clustered together in the haplotype network (Fig. 33), certain geographical discontinuities can be observed. The barrier that disconnects populations of Croatia and Montenegro from other localities could be explained by hydrographic isolation of the Adriatic Sea associated with the Otranto Strait which separates the Adriatic Sea from the Mediterranean. This is not surprising, as many studies show genetic differentiation between these two water masses (Boissin et al., 2011; Borsa et al., 1997; Rossi et al., 2006). Additionally, since Sicily and France were estimated by Geneland to create one population, the Sicily Channel could not pose the barrier in this case. It is hard to say what oceanographic discontinuity could cause gene flow limitations between these two areas. However, the Aegean front and local circulation mechanisms might be the possible explanations for the partial genetic isolation (Theocharis et al., 1999). To examine this, samples from multiple locations in the given area would be needed. According to our own observations, this species is quite common in central and eastern Mediterranean Sea, however, it is very rare in the western part.

Another interesting finding was in the case of another epibenthic species, *G. incognitus,* where a great variability of haplotypes (81 haplotypes) has been recognised for

cyt b (Fig. 38). There are mostly unique haplotypes with only six haplotypes shared between two to five specimens. The haplotype network did not exhibit any geographic pattern, but Cyprus appeared to have the least related populations with that from the other sites (separated by DAPC) (Fig. 39). The result of Geneland (Fig. 44) was a rather patchy structure, which does not provide any solid explanation about the population subdivision. Such great haplotype variability suggests a large population of *G. incognitus*.

Absence of genetic structure has been found in *C. liechtensteini* for cyt b (Fig. 20). Zero F_{ST} index indicated panmictic population what was also demonstrated by DAPC (Fig. 21) and Geneland (Fig. 27), which identified all localities as one big population. The genetic distances were low (0.02 - 0.2%) (Tab. 12), both between and within the subpopulations. Haplotype network, with a 'star-like' structure, showed one large central haplotype and several unique haplotypes, of which most were closely related to the common central haplotype. Dominant haplotype was shared between all geographically distant areas, except of France. This is an interesting fact as *C. liechtensteini* is a cryptobenthic species and migrations of adults are considered to be considerably limited. *Corcyrogobius lichtensteini* is a cryptobenthic species, hidden in small cavities. It can be locally quite abundant, but it has special requirements for microhabitat. As all cryptobenthic species, it is very difficult to collect it.

In other cryptobenthic species, although some structure or gradient was revealed in some cases, the number of samples and geographic coverage was very low, to be able to draw any conclusions. In the case of *Chromogobius zebratus* sp. 1, the subpopulation from Portugal seems to be divergent from the other subpopulations, suggesting that Gibraltar strait might be a barrier. Also, Sicilian channel might be another important barrier for migration and gene flow of this species.

Apart from the mitochondrial gene cyt b, also the first intron of ribosomal protein gene S7 was analysed. In fishes, this nuclear marker is in general very variable, so it has been used also for studies of phylogeography or population structuring (Durand et al., 2013; Gilles et al., 2000; Gysels et al., 2004a; Lemaire et al., 2005). Patterns of population variation and differentiation of nuclear and mitochondrial marker differ significantly, which is not surprising. However, the polymorphism of S7 in my dataset was reduced due to presence of indels, by cutting the alignment at the beginning of the first indel. In some cases, the phasing of several sequences was not reliable, so these were needed to be removed from the dataset prior to the analyses; this also reduced the final polymorphism of the analysed dataset. Anyway, I do not expect that the lost polymorphism would have contributed to some geographical pattern of genetic diversity of the studied species.

Discrepancies between mitochondrial and nuclear markers are common also in other phylogeographic and population genetic studies (Boissin, 2016; Limborg et al., 2012; Wilson

and Eigenmann Veraguth, 2010). Due to practically no variability observed in *G. cruentatus* and *G. geniporus* for S7, some analyses were not performed; complete data analyses in S7 were done only in two species, i.e. *C. liechtensteini* and *G. incognitus*. In both these species polymorphism in S7 was big. Surprisingly, the polymorphism of S7 was higher than that of cytochrome b in one out of four species. This greater variability was found in the case of *C. liechtensteini*.

In general, mitochondrial markers are more informative for population genetic studies than nuclear ones (with the exception of microsatellites). Mitochondrial gene cyt b is one of the most widely used DNA markers in fish genetics. The fact that cyt b is more informative than S7 is due to its high level of polymorphism, this is owned to a high mutation rate, and associated faster evolution (Lee et al., 1995; Wilson et al., 1985). The length of sequences for cyt b was in the range 1080 - 1117 base pairs while for S7 it was sometimes less than half of it (352 - 602 bp). The advantage of combining multiple markers for studies of population structure and phylogeography have already been presented in several marine fishes (André et al., 2011; Gonzalez and Zardoya, 2007; Wilson and Eigenmann Veraguth, 2010). For the comparison of population genetic diversity, microsatellites are much more suitable than other nuclear markers (Gonzalez and Zardoya, 2007).

A greater haplotype diversity of S7 (32 haplotypes) than that of cyt b (18 haplotypes) was found in *C. liechtensteini*. Much higher polymorphism in nuclear marker S7 than in mitochondrial marker cyt b in *C. liechtensteini* might be explained by the sudden decline of population which previously had much higher genetic diversity, and its subsequent dispersal. Another explanation might be that the population recently dispersed from a refugium. In both cases, there must have been a restriction in mating, i.e., only females from one restricted subpopulation were able to reproduce, or males, unlike females, were able to migrate (e.g. from the other refugia). Another hypothesis is that mitochondrial introgression by another species occurred in the past. Analyses of more nuclear markers, preferably by some of the methods of the next generation sequencing, might give some clue to this puzzle.

Genetic diversity and demographic history

High haplotype diversity and moderate nucleotide diversity observed for cyt b in all species, are congruent with the values recorded in other marine fishes (Charrier et al., 2006; Shulman and Bermingham, 1995; Viñas et al., 2004; Zardoya et al., 2004). Our results indicate population expansion from a small size and rapid growth enhancing the retention of new mutations (Avise et al., 1984; Charrier et al., 2006). This was further supported by negative values of neutrality tests (Tajima's D, Fu and Li's). The Bayesian Skyline plot for cyt b marker shows a period of population expansion in the past and stable population in recent. Such scenario could be the result of fluctuations during the Pleistocene glaciations when colonisation and population regressions and expansions occurred (Hewitt, 2000). Since times

to the most recent common ancestor were not estimated we cannot suggest when the expansion began. This should be ideally done in further studies. Bayesian Skyline plot for S7 was constructed only in two species, while the pattern, matching to cyt b, was the same only in the case of *G. incognitus* (Fig. 43) and in *C. liechtensteini* (Fig. 25)was different. This is due to different evolution of these two genes. Nuclear genes evolve much more slowly than mitochondrial ones. Low values of haplotype and nucleotide diversity correspond to very low variability of S7 in *G. cruentatus* and *G. geniporus*. Relatively high nucleotide diversity (π = 0.015) in *C. liechtensteini* for S7 (see Tab. 7) ranks among the highest reported within studied species. This would suggest that it is the large population with stable effective size, as there has been sufficient evolutionary time for new mutations (Giovannotti et al., 2009).

Hidden diversity – complexes of species

Several new species of gobies have been discovered and described recently from the Mediterranean Sea (Engin and İnnal, 2017; Kovačić and Šanda, 2016; Kovačić et al., 2016, 2017). This shows that the diversity of European marine gobies is still not completely known. In my MSc thesis I have discovered hidden diversity within several species. One of the revealed cases of higher diversity was in the *Chromogobius zebratus* species complex, where three lineages were discovered within the dataset. Both Bayesian and Maximum Likelihood phylogenetic trees supported well all three lineages and the close relatedness between the groups has been proved. Interspecific genetic distances between the three species were high, with 12 - 20% divergence (Tab. 36).

Second revealed complex of species was in the case of the clade comprising *Millerigobius macrocephalus* and *Zebrus zebrus* where one more lineage was discovered within the dataset (referred here to as *Zebrus* cf. *zebrus*). Interspecific genetic distances on cyt b between the three species were 23 - 25% (Tab. 42). Within two of the species even greater variability was found, a divergent clade being represented in each case just by one specimen. The sample MillerMC was distant by 3.7% from *M. macrocephalus* and the sample H1723 by 5.3 % from *Zebrus* cf. *zebrus* (Tab. 41). It is not clear whether there is much greater intraspecific variability or more cryptic lineages exist; to resolve this, more samples would be needed.

As both complex *Chromogobius zebratus* and *Zebrus* - *Millerigobius* include closely related and morphologically similar species, determination of species is very difficult. To assign these newly discovered lineages to species, detailed morphological examination of the type material and our material have to be undertaken. It is also not excluded that the complexes encompass even more species and additional cryptic species will be discovered, as more material will be analysed.

In both cases, these are cryptobenthic species, hidden in substrate or in cavities and are very small (max size 6 cm) (Patzner, 1999). They are very difficult to find and to collect.

Practically, without the use of anaesthetic, it is almost impossible even to observe them. That is why it is so difficult to obtain sufficient material size and from many localities.

There are morphologically cryptic species among European marine gobies whose existence has been detected by DNA analysis. This was the case of *Gobius incognitus*, which was for a long time considered *Gobius bucchichi*, a species very similar in morphology and ecology (Kovačić and Šanda, 2016). Another case is *Pomatoschistus minutus* complex, where individuals from the northern part of the Adriatic Sea are genetically very distinct from the populations of the western Mediterranean and the Atlantic Ocean (Boissin et al., 2011; Gysels et al., 2004c; Stefanni and Thorley, 2003; Stefanni et al., 2003). However, the taxonomy of an apparent *P. minutus* complex is not resolved to date. On the other hand, it is known that there are also complexes of closely related species, e.g. so called yellow gobies (*Gobius auratus, Gobius fallax* and *Gobius xanthocephalus*), which are morphologically very similar to each other, so their identification is very difficult. In the case of this species complex, a great genetic similarity has also been demonstrated, since *G. auratus* and *G. fallax* cannot be distinguished by using mitochondrial DNA (Herler et al., 2005). In my study the presence of three unknown species was revealed with the use of mitochondrial marker.

In my thesis, in the two complexes of species, closely related groups of species were found to inhabit the same localities in several cases (two or even three species occurring in sympatry). It can be speculated that sympatric speciation events might have occurred. Boissin et al. (2011) suggested that speciation happens more likely in the fishes with short life cycles and fishes inhabiting very unstable habitats. For studied species, only the first statement is relevant as average life-span of gobies is one or two years (Hwang and Baek, 2013). Sympatric speciation is a process which might happen in the absence of geographical barriers and hence it is not very common in vertebrates (Coyne and Orr, 2004). Although speciation under sympatric conditions is theoretically possible, there are only a few relevant examples of sympatric speciation, e.g. cichlids from the African and Central American lakes (Barluenga et al., 2006; Fruciano et al., 2016; Kautt et al., 2016; Meier et al., 2017). In African lakes, species flocks of cichlids, of ancient hybrid origin were revealed (Meier et al., 2017). If the speciation in our two species complexes was due to ancient hybridisation or not, can be speculated, but it would be very difficult to prove. In any case, nuclear markers need to be analysed in these species, as well as morphology to be able to confirm their status as species. A high-flown aim would be to study their ecology, but this is rather impossible, at least at present.

Migrations and passive planktonic dispersal

The possible explanation for the genetic differences in the studied populations, are different dispersal capabilities. Migrations in adult gobies have been reported only in few species. However, it concerns short-distance migrations in the mating season to warmer or

nutrient-rich waters. It is assumed that these migrations are performed to find better conditions for the embryonic development of offspring or for freshly hatched larvae (Boissin et al., 2011; Iglesias and Morales-Nin, 2001; Pampoulie, 2001). However, no other significant migrations have been documented yet. Thus, the spatial distribution of genetic variability depends mostly on passive transport of larvae (Stefanni et al., 2003). It seems that planktonic larval duration is one of the main factors influencing homogenisation of the population structure (Shanks et al., 2003). Planktonic life stages have variable duration of larval period in gobies (Akihito et al., 2000), but usually last about four weeks (Planes, 1998). Dispersal capabilities of marine organisms are influenced by currents and other oceanographic processes. This allows them a long-distance dispersal and gives them high potential for gene flow (Patarnello et al., 2007; White et al., 2010).

Concluding remarks

Important drivers which have impact on population genetic connectivity are oceanographic barriers and discontinuities. My work assessed the possible influence of the Mediterranean barriers on the genetic structure of several species of gobies, but none of them were confirmed with certainty. The Strait of Gibraltar and the Almeria-Oran front, which play a significant role in the differentiation of several species, such as *P. microps* (Gysels et al., 2004a), common sea bass, *Dicentrarchus labrax* (Lemaire et al., 2005), European anglerfish, *Lophius budgassa* (Charrier et al., 2006) and others, in my study did not separate the Atlantic populations from the western Mediterranean populations. On the contrary, according to the results of *G. cruentatus* and *G. geniporus*, it seems that the genetic break occurs in the western central Mediterranean what was also the case of the rusty blenny, *Parablennius sanguinolentus* (Pappalardo et al., 2017). This suggests, that the existence of biogeographical barriers that have hindered or still hinder migration between areas. In extreme cases, the isolation is so long that it leads to formation of a multi-species complex. To sum up, both historical and contemporary hydrographic regimes play an important role in the marine environment. Main processes influencing genetic differentiation in Mediterranean Sea seem to be explained by the combination of the marine currents, oceanographic barriers and some specific environmental conditions (Galarza et al., 2009b; Schunter et al., 2011).

The diversity in cytochrome b was high in the most studied species. A larger sample size and / or samples from more localities, especially in the case of cryptobenthic species could provide more detailed picture of the population subdivision and existence of possible barriers of species dispersion. Moreover, there is a complete lack of knowledge about the dispersal and duration of larval stages.

Summary

Overall, my Msc thesis was focused on the comparison of genetic variation in geographically distant populations of eight species of European gobies *(Corcyrogobius liechtensteini, Gobius cruentatus, Gobius geniporus, Gobius incognitus*, *Chromogobius quadrivittatus, Chromogobius zebratus, Millerigobius macrocephalus* and *Zebrus zebrus*). In this study I have included samples from two Atlantic localities (Spain and Portugal) and seven localities from the Mediterranean Sea (France, Sicily, Croatia, Montenegro, Greece, and Cyprus - northern and southern part). This work is based on molecular analyses of the mitochondrial gene cytochrome b as well as the first intron of the nuclear ribosomal protein gene S7. The patterns between the markers differed significantly. Cyt b was more polymorphic in all species, except of *C. liechtensteini*, where S7 was more polymorphic. A combination of both markers has shown to be useful.

Certain degree of genetic partitioning and potential existence of geographic barriers was found in *G. cruentatus* and *G. geniporus*. In case of *G. cruentatus*, genetic structure was discovered between the western and eastern part suggesting that Sicily Channel could play a role in this differentiation. Distribution of *G. geniporus* was divided into three sub-regions such as western, eastern and northern central Mediterranean. Some of the possible barriers in this case could be the Otranto Strait and Aegean front. Although some geographic discontinuities were observed, the results should be taken rather provisional and more detailed sampling would be needed. No clear geographic pattern was revealed in the remaining species. Interestingly, low genetic variability in cytochrome b was found in *C. liechtensteini,* which is cryptobenthic species and thus its migrations in adult stage are very limited.

Furthermore, I have discovered a hidden diversity within two clades of cryptobenthic species. Three unknown species were found. One case was that of *Chromogobius zebratus* complex, where two new subclades were found, with the genetic distance between subclades on cytochrome b of 12 - 20%. Second revealed case with one newly discovered subclade was in the clade comprising *Millerigobius macrocephalus* and *Zebrus zebrus.* The genetic distance on cytochrome b was 23 - 25%. Moreover, the results indicate that even greater diversity is present in these complexes of species, for which confirmation a dense sampling would be necessary, as the closely related subclades often live in sympatry. For species classification a detailed morphological examination has to be undertaken.

As no significant migrations have been documented yet in adult stages of gobies, larval planktonic stages are probably more important for the genetic structure of populations of gobies than we thought. They passively migrate in the sea, influenced by currents and oceanographic barriers. Known geographic breaks, such as the Strait of Gibraltar and the Almeria-Oran front, were not confirmed to be important in my work. Last, genetic differentiation between populations in the sea is the result of the combination of different past and present hydrographic processes and the traits of organisms.

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