Univerzita Karlova

Přírodovědecká fakulta

Studijní program: Biologie
Studijní obor: Ekologie

Bc. Jakub Hradečný

Fylogeografie a genetická diverzita populací druhu *Gobius niger* (Gobiiformes: Gobiidae)

Phylogeography and genetic diversity of the populations of *Gobius niger* (Gobiiformes: Gobiidae)

Diplomová práce

Školitelka: RNDr. Jasna Vukić, Ph.D.

Praha, 2023

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 zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 5. ledna 2023

Jakub Hradečný

Acknowledgments

On the first place, I would like to thank to my supervisor, Jasna Vukić, for her professional leading. I am very thankful for her patience, understanding and willingness to help whenever it was necessary.

I would also like to thank to Joana Robalo, for the opportunity to realize an internship and collaborate during the hard, pandemic times.

Finally, I would like to express my gratitude to my family, supporting me in my interests in nature since childhood.

Abstrakt

Hlaváči (Gobiiformes: Gobiidae) jsou nejpočetnější rybí čeledí a nejvíce zastoupenou skupinou ryb obývající evropské pobřeží. Genetická diverzita evropských hlaváčů byla doposud studována pouze u několika málo druhů. Ačkoli se mořské prostředí jeví jako homogenní, existují zde bariéry bránící genovému toku, například v podobě průlivů či mořských proudů.

Hlaváč černý (*Gobius niger*) obývá pobřežní vody Evropy, severní Afriky a také středozemní a černomořské pobřeží Blízkého východu. Jedná se o bentický druh hlaváče, který má v rámci životního cyklu planktonní larvální stadia. V rámci této práce byli studovaní jedinci z 28 lokalit napříč areálem rozšíření, s cílem studia vnitrodruhové genetické diverzity. Analýza dvou mitochondriálních genů (cytochrom b a D loop) odhalila komplexní populační strukturu a výraznou vnitrodruhovou diverzitu (maximální p-distance 6,1 %). Populace je rozčleněna na čtyři velmi rozdílné haplotypové skupiny, s neočekávaným geografickým rozložením. Zatímco populace obývající Atlantský oceán a Severní moře je geneticky homogenní a všichni studovaní jedinci nesou haplotypy patřící do stejné haplotypové skupiny, středomořská populace je velmi komplexní, a vykazuje přítomnost čtyř haplotypových skupin, z nichž některé jsou sdílené i mezi vzdálenějšími regiony. V západním středomoří a v Jaderském moři byla zjištěna přítomnost tří odlišných haplotypových skupin, dokonce i v rámci jedné lokality, což indikuje sekundární kontakt alopatrických linií.

Populace z různých regionů vykazovaly výrazné odlišnosti, s výjimkou podoblastí v rámci Atlantského Oceánu a severního moře, kde byla diferenciace mezi populacemi nízká či střední. Konektivita mezi populacemi hlaváče černého se zdá být silně ovlivněna hydrologickou strukturou, což odpovídá bentickému způsobu života dospělců a dlouhé době pelagického larválního stadia. Přítomnost odlišných haplotypových skupin v severním a jižním Jaderském moři svědčí o nízké konektivitě mezi oběma oblastmi. Populace z jižního Jaderského moře je naopak propojena s populací z Iónského a Egejského moře, a Otrantský průliv tedy zřejmě nepůsobí jako bariéra genového toku mezi populacemi hlaváče černého. Oproti tomu Sicilský průliv a Turecké průlivy Bospor a Dardanely výrazně redukují konektivitu mezi populacemi hlaváče černého. Naopak přítomnost několika sdílených a dalších velmi podobných haplotypů mezi západním Středomořím a Atlantským oceánem podporuje hypotézu o existující konektivitě mezi populacemi skrze Gibraltarský průliv.

Klíčová slova:

hlaváč, mořské ryby, Středozemní moře, molekulární data, populační struktura

Abstract

Gobies (Gobiiformes: Gobiidae) are the most speciose fish family and the most abundant fish group of the European seas. Yet, the population genetic diversity has been studied in only a few European goby species. Although the ocean is seemingly a continuous habitat, there are barriers to gene flow in marine environment in form of e.g. straits or marine currents.

The black goby (*Gobius niger*) is a demersal marine fish with planktonic larvae, inhabiting the coastal waters of Europe, north Africa and the Mediterranean and Black Sea coast of the Middle East. The current work examined individuals from 28 localities across the distribution range of the species in order to study the population genetic diversity. The analyses of two mitochondrial markers (cytochrome b and D loop) revealed a complex structuring of the population and a pronounced intraspecific diversity (maximum uncorrected p-distance 6.1 %). The population is divided into four very diverse haplotype groups, with unexpected geographic pattern. While the population inhabiting the Atlantic Ocean and the North Sea is generally genetically homogeneous, with all examined individuals bearing haplotypes belonging to a single haplotype group, the Mediterranean population is very complex, displaying a presence of four haplotype groups, some of which shared between the non-neighbouring regions. In the western Mediterranean Sea and in the Adriatic Sea, the presence of three distinct haplotype groups was uncovered even at a single locality, indicating a secondary contact of allopatric lineages.

The differentiation of the populations from different regions was pronounced, with the exception of the subregions within the Atlantic Ocean and the North Sea, where the differentiation between the populations was in most cases low to moderate. The connectivity between the populations of the black goby seems to be highly influenced by the hydrological patterns, which is in accordance with the demersal non-migratory life style of adults and long pelagic larval duration. A very low connectivity between the populations from the northern and southern Adriatic Sea basin is suggested from the presence of different haplotype groups in each part of the basin, which is caused by a complex circulation within the basin. Moreover, the population from the southern Adriatic Sea seems to be connected with the populations from the Aegean and Ionian seas, indicating that the Otranto Strait does not act as a barrier for gene flow between the populations of the black goby. On the other hand, the Sicilo-Tunisian Channel and the Turkish strait system apparently reduce the connectivity between the populations of the black goby. The presence of several shared and other highly similar haplotypes between the western Mediterranean and the Atlantic populations supports the hypothesis of the existence of population connectivity through the Strait of Gibraltar.

Key words:

goby, marine fish, Mediterranean Sea, molecular data, population structure

Contents

1: Introduction

1.1: Connectivity in marine environment

Populations of marine organisms were recognised for a long time as open and well connected (Warner & Hughes, 1988), until the fall of this paradigm at the end of last century which came together with broader use of molecular technics in the study of biogeography and phylogeography (Cowen et al., 2000; Cowen & Sponaugle, 2009). The molecular studies revealed that genetic structure exists for many marine organisms, and that there are barriers in the sea preventing the gene flow between metapopulations (Palumbi, 2003; Galarza et al., 2009). The gene flow within metapopulations is a result of migration of individuals, which, for the benthic sedentary organisms with demersal eggs means mainly the transport of the larvae by water currents as a part of zooplankton. The potential connectivity is dependent on the length of the time spent in the mobile phase, so called planktonic larval duration (PLD) (Selkoe & Toonen, 2011) and on the past and present hydrological characteristics. In many fish species the eggs can be also released to the water column and thus they are also recognised as a dispersal unit. The length of the PLD is traditionally measured by counting the growth rings of the otoliths of lately settled juveniles (Raventós & Macpherson, 2001).

Once in the water column, it is very hard or even impossible to track the movement and behaviour of the larvae (Leis et al., 2006). Predictions of the possible larval dispersal in the open ocean are modelled as a track of virtual lagrangian particles. The first models were done as a simple tracking of the particle carried by the water currents (Gilg & Hilbish, 2003; Siegel et al., 2003), but these models usually underestimate the different hydrology of the shallow coastal waters (Nickols et al., 2015), which can be crucial for the dispersal of the coastal benthic organisms (Woodson et al., 2012).

Other aspect of dispersal, which used to be overlooked in the past, is larval behaviour. The behavioural traits of the larvae of different benthic organisms have been tested in laboratory, and they can have determinative effect on the population connectivity (Shanks, 2009) because the planktonic larvae are not just passively drifted by currents, as used to be assumed in the past. As proved, some larvae show phototactic behaviour (Faillettaz et al., 2015), and as the fish larvae of many species (including the Black goby) do have proportionally big eyes, the good visual capacities are expected (Leis & Carson-Ewart, 2000). So far discussed is the end of the planktonic phase and the settling of the juveniles to the bottom. In the enhanced lagrangian models, the virtual particles tracked also presented behaviour, as in (Lacroix et al., 2012), where the virtual larvae show positive geotaxis when approaching the coast close enough to detect the bottom visually. Using these models, we are approaching to be able to predict the dispersal capacities of species, once we will know their biological characteristics. The models are good for description of possible connectivity under actual hydrological situation, but even enhanced models should be calibrated by real genetic data which can confirm the hypotheses (Selkoe et al., 2016). Compared to the dispersal simulations, using molecular methods we are able to detect not only the actual connectivity, but also the dispersal and population dynamics which occurred on the scales of years to millions of years in the past (depending on the molecular marker used)(Habib et al., 2011).

1.2: Hydrological structure of the area inhabited by the black goby

Figure 1: The distribution of the *Gobius niger* (IUCN 2022)

As described in 1.1, both historic and actual hydrological patterns have a crucial effect on the evolution of the species inhabiting the coastal ecosystems (Selkoe et al., 2008). In the following part, the summary of hydrological system of the area inhabited by the black goby is described with the focus on the explanation of possible biogeographic barriers. The black goby is distributed across the coasts of the Mediterranean Sea, the Black Sea and the North-eastern Atlantic, from the northern Mauritania to Norway (northernmost documented distribution is near the city of Trondheim (Fries et al., 1892)), including Macaronesian archipelagos (except Azores), British islands and the western parts of the Baltic Sea (see the Fig. 1) (Vaas et al., 1975; Nash, 1984; Claridge et al., 1985; Vesey & Langford, 1985; Miller, 1986; Magnhagen, 1988; Filiz, 2013; Kara & Quignard, 2019). For closer description of the circulation patterns, the area inhabited by *Gobius niger* Linnaeus, 1758 has been divided into following several hydrologically distinct regions: the Black Sea, the Mediterranean Sea, the Atlantic Ocean and the North Sea.

1.2.1: The Black Sea

The semi-enclosed basin which lies on the border between Europe and Asia belongs among one of the most isolated marine systems in the world. It is connected with the Mediterranean Sea through the Turkish strait system, consisting of the narrow straits of Bosporus and Dardanelles with the Marmara Sea in the middle. From the other side, the Black Sea is a sink of several major European rivers (e.g. Danube, Dnieper, Dniester). The high input of the freshwater together with limited connection to the Eastern Mediterranean Sea resulted in low salinity of the basin varying between 17.5 – 18 ppm in the open sea (Zaitsev, 2008).

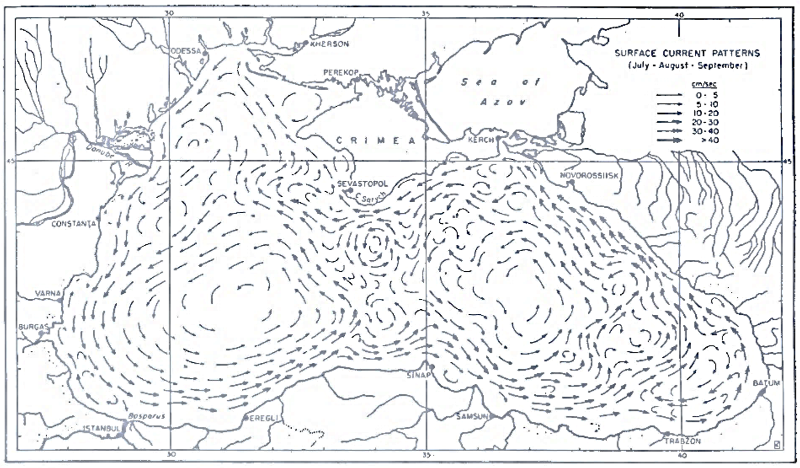

Figure 2: Current chart of Black Sea showing midsummer surface currents patterns (Zenkevich, 1963)

The surface water circulation is dominated by the counter-clockwise current (Fig. 2). Moreover, in the narrowest part in the middle of the Black Sea basin, between the Crimean Peninsula and the Anatolian coast, a part of the current flowing from the west turns north, dividing the basin into two semi-separated circulatory systems (Zenkevitch, 1963). Apart from this main circulation pattern, some smaller more or less stable cyclonic and anticyclonic circulation bodies can be found (Fig. 2).

The connection of the Black Sea with the Mediterranean has not been historically stable. There is an evidence that during the Pleistocene climatic oscillations, the two basins were separated during the dry glacial periods, and that the connection with the Mediterranean Sea re-established at least twelve times in the last 670 000 years as the sea level changed (Badertscher et al., 2011). Last time, the reconnection of the two basins occurred at the end of the last glacial maximum, approximately 11 500 – 11 100 years ago, with gradual penetration of brackish waters from the former Neoeuxin lake (low salinity stage of the Black Sea) to the northern Aegean Sea followed with establishment of the deeper opposite-way current of the Mediterranean saline water flowing northward to the Black Sea (Aksu & Hiscott, 2022).

The turbulent oscillations in the sea level and salinity affected the demographic history of marine inhabitants. Several phylogeographic studies proposed that the Black Sea has been inhabited by euryhaline fish species (*Hippocampus guttulatus* Cuvier, 1829 (Woodall et al., 2015) or *Sprattus sprattus* Linnaeus, 1758 (Debes et al., 2008)) during the last glacial maximum, and that the episodic colonisation and isolation followed with renewed gene flow are responsible for the high genetic variability comparable to Eastern Mediterranean (Debes et al., 2008; Woodall et al., 2015;). On the contrary, the Black Sea was probably unfavourable for marine species with higher salinity requirements like for example *Scorpaena porcus*

Linnaeus, 1758 (Boissin et al., 2016) or *Engraulis encrasicolus* Linnaeus, 1758 (Magoulas et al., 1996), which show indications of recent population expansion within the Black Sea basin. The narrow connection to the Mediterranean Sea was described as possible barrier to the gene flow for some marine organisms (Durand et al., 2013; Şalcıoğlu et al., 2020), although for organisms with different life-history characteristics the detection of the barrier failed (Kalkan et al., 2011).

Moreover, the study of Turan et al. (2009) does not describe the Turkish strait system as a barrier for the Atlantic horse mackerel (*Trachurus trachurus* Linnaeus, 1758), but the authors have found differences between the populations from the western and eastern Black Sea. The population heterogeneity of the population of turbot (*Scophthalmus maximus* Linnaeus, 1758) shows that barriers to the gene flow can be present within the basin (Firidin et al., 2020). Since the area of the Black Sea still remains poorly studied, our knowledge about the biological connectivity within the region is limited and high sampling effort is necessary to evaluate correctly the degree of population connectivity.

1.2.2:The Mediterranean Sea

As the name of this water body indicate, the Mediterranean Sea is almost completely enclosed by lands of the Southern Europe, North Africa and the Middle East. The only natural opening to the world ocean system is the Strait of Gibraltar, which enables the inflow of the Atlantic waters to the basin. Moreover, the anthropogenic Suez Channel connects the eastern Mediterranean with the Red Sea and thus enables the entrance of the Indopacific fauna to the Atlantic-Mediterranean system.

The determinative characteristics of the Mediterranean Sea is the negative water balance, as the freshwater input from the major Mediterranean rivers (Nile, Po, Rhône, Ebro) and through the precipitation is exceeded by the evaporation (Millot & Taupier-Letage, 2005). Because of the high evaporation rates, for the Mediterranean Sea is typical high salinity, rising in the southeast direction from the 36 ppt in the Alboran Sea up to the 39.5 ppt in the Levantine Sea (Kovačić & Patzner 2011). This water loss is being balanced with the inflow of the less saline Atlantic waters through the Strait of Gibraltar. On the contact of the inflowing less saline Atlantic waters and the concentrated warm Mediterranean Sea, the two water masses do not mix, but the highly dense Mediterranean waters are forced to detrain so they can be overflown by the Atlantic waters. The narrow contact zone between the two water masses presents a steep gradient of temperature and salinity known as Almeria-Oran front (Fielding et al., 2001; Zarokanellos et al., 2022).

The Mediterranean Sea can be divided into the two parts, the western and eastern basins, which are connected through the Siculo-Tunisian channel. Due to the complex geomorphology, only main currents and fronts important to the biogeography of the area will be described (see Fig. 3).

In both western and eastern basins, the dominating current flows in the counter-clockwise direction as a result of the Coriolis effect. The former-Atlantic water entering from the Alboran Sea follows the coast of North Africa in the eastern direction as so-called Algerian Current and Libyo-Egyptian Current. This current is not stable, and it is frequently interrupted by local and temporary anticyclonic eddies (Millot & Taupier-Letage, 2005) which can accumulate part of the Atlantic water in the southern subbasins (Barral et al., 2021). When passing through the Sicilo-Tunisian channel, a part of the current branches northward to the Tyrrhenian Sea, following the western coast of Italy up to the Ligurian Sea. In the North of the Western Mediterranean basin, the current remains permanently stable, following the coasts of France and Spain (Millot, 1999). Reaching the south of the Balearic Sea, a part of the current branches and turns back in the north-east direction, as a result of orographic conditions in the southern Balearic Sea basin together with the salinity differences between the northern and southern subbasins. On the contact of two masses, the colder and more saline waters of Mediterranean Sea in the north with the less saline and warm former-Atlantic waters creates the Balearic front (Millot, 1999; Millot & Taupier-Letage, 2005). This front arises as a result of the salinity differences, whereas the temperature differences between the two water masses

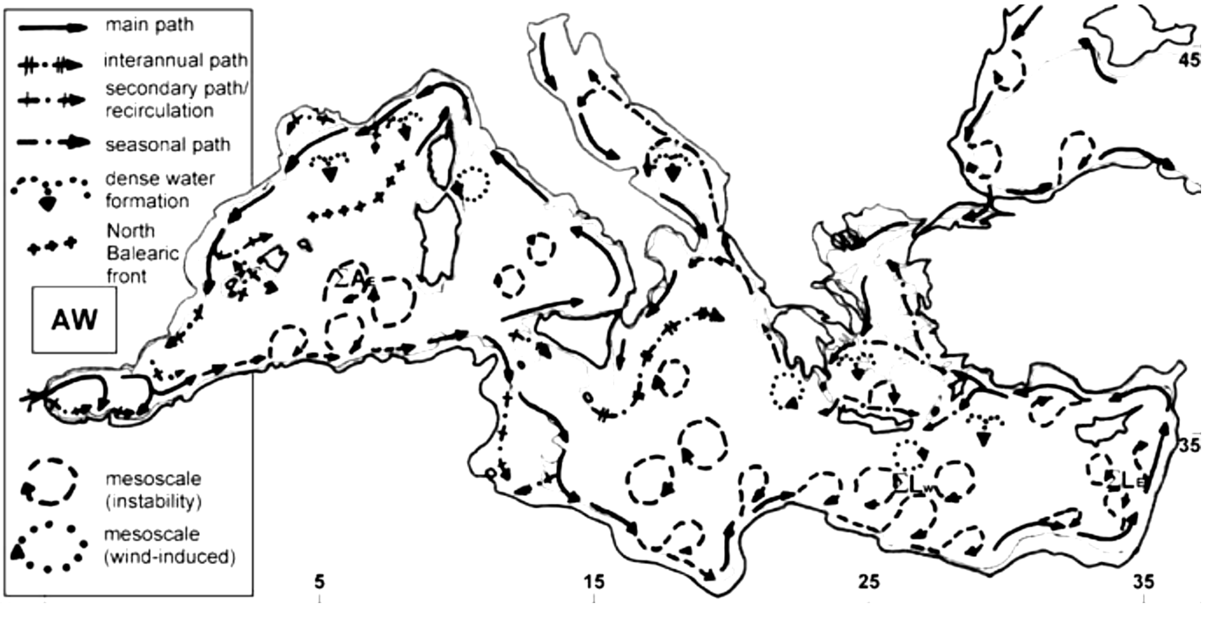

Figure 3: The main circulation patterns in the Mediterranean basin. The thin line represents the 200 m isobath (Millot & Taupier-Letage, 2005)

changes during the year, as the northern subbasin experience deep cooling and mixing episode in the winter period (Pinot et al., 2002; Barral et al., 2021).

In the Eastern basin of the Mediterranean Sea, the two major components of the circulation can be named. In the southern part, as mentioned in previous paragraph, the main flow is represented by the unstable Libyo-Egyptian current passing along the coast of Africa towards the Middle East (Hamad et al., 2006).

In the northern part of the Eastern Mediterranean basin, the hydrological situation becomes more complicated due to the orographic complexity of the area. In the Aegean Sea, the general circulation has counter-clockwise character, with the main current flowing on the rim of the subbasin (Hamad et al., 2006), see the Fig. 3. Important hydrological structure is located in the northern Aegean Sea, where the low-saline waters from the Black Sea are penetrating the Aegean Sea in the Dardanelles plume region. The outflow from the Dardanelles continues in the western direction, which is enhanced by the etesian winds that impact the eastern Aegean Sea during summer and autumn (Olson et al., 2007). Once reaching the western rim of the Aegean subbasin, strong southward current is established and exits the Aegean Sea through the western Cretan Arc (Millot & Taupier-Letage, 2005; Hamad et al., 2006; Olson et al., 2007).

The remaining part of the Eastern Mediterranean basin is being sometimes recognised together with the Aegean Sea as a Central Mediterranean, which besides includes the Ionian and the Adriatic subbasins. The Ionian Sea presents very complex hydrology, with a numerous temporal eddies propagating from the main cyclonic current (Hamad et al., 2006). Apart from the main current flowing from the southeast direction, the Ionian Sea can be enriched by the inflow of the southern current branch when passing the Sicilo-Tunisian Channel. The system is thus very unstable with occasional abruptions leading to the regime shifts in the general circulation patterns (Hamad et al., 2006; Kalimeris & Kassis, 2020). On the contrary, the Adriatic subbasin is typical with highly stable and permanent cyclonic circulation (Kalimeris & Kassis, 2020). The Adriatic Sea circulation is shaped by the annual climatic oscillations as well as by the considerable freshwater inflow from the Po river. Those factors are responsible for the division of the subbasin into the northern and southern parts (Hariri, 2022). Moreover, the southern part of the subbasin can be temporarily divided into the two separated gyres, as described by Artegiani et al. (1997).

1.2.3: The Atlantic Ocean and the North Sea

The circulation within the north-east Atlantic is highly influenced by the strong current originated in the area of greater Caribbean region, flowing in the north-east direction throughout the north Atlantic Ocean (Centurioni & Niiler, 2003). When reaching the latitude of approximately 40° N, the current branches into two streams, the North Atlantic current following the north-east direction, and the Azores current turning the direction to the east (Klein & Siedler, 1989), dividing the subtropical and temperate water masses of the North Atlantic (Bonfardeci et al., 2018).

The Azores current continues in the eastward direction, reaching the northern coast of Africa, where the current ramifies. The north branch is entering the Mediterranean Sea through the strait of Gibraltar, whereas the southern branch follows the African coast in the southward direction (Klein & Siedler, 1989; Bonfardeci et al., 2018).

Another division of the North Atlantic current appear approximately on the 50°N latitude, where the Portugal current follows the eastward direction approximately to the 20°W, where the current turns southward following the western coast of the Iberian Peninsula (Bonfardeci et al., 2018). The Portugal current is the dominating force in the Iberian basin, but due to the influence of the Azores current, the opposite Iberian Poleward Current is generated flowing northward closely to the Iberian coast (Peliz et al., 2005). This poleward current is supposed to be persistent all year round (Relvas et al., 2007). In the Bay of Biscay, the circulation patterns differ significantly during the year, having the clockwise direction at summer and turning to counter-clockwise direction during the winter (Charria et al., 2011).

In the North Sea basin, the circulation has general counter-clockwise character, which is affected by the entrance of the Atlantic water from the north (Turrell, 1992).

1.3: The history of the Mediterranean

The evolution of the biota occupying the Mediterranean Sea is tightly dependent on the historical events changing the climate and the hydrological patterns in the past (C. Nike Bianchi & Morri, 2000). Probably most important geological event directly affecting the evolution of recent genera is supposed to be the Messinian salinity crisis (Hsü et al., 1973), the desiccation event caused by the proposed closure of the Atlantic-Mediterranean connection which resulted from the tectonic activity between 5.96 and 5.33 million years ago (Duggen et al., 2003). For the long time it has been postulated that the Mediterranean had become converted in the isolated, hypersaline lake, which led the former Atlantic-Mediterranean species to extinction or speciation. The Mediterranean area was therefore re-invaded by the Atlantic species once the connection between the two systems was re-established at the end of Miocene (Kovačić &

Patzner 2011). Once the newly flooded (Garcia-Castellanos et al., 2020) Mediterranean Sea was re-invaded, it is proposed that the high number of the empty ecological niches might have led to the species radiation (Ahnelt et al., 1995; Huyse et al., 2004). The theory of huge desiccation event followed by re-invasion from the Atlantic Ocean is being criticized as the new paleonthologic research revealed possible existence of stable marine environment during the proposed salinity crisis (Carnevale & Schwarzhans, 2022) with a diverse assemblage of gobioid fauna (Schwarzhans et al., 2020).

The present-day distribution of contemporary fish species has roots in the more recent events, especially the climatic oscillations during the Pleistocene (C. Nike Bianchi & Morri, 2000; Tougard et al., 2021) leading to the alteration in the sea level (Rabineau et al., 2006) and thus affecting the potential distribution of the species. The climatic changes influenced also the intensity of the marine currents (Flores et al., 1997) which do play important role in the connectivity of marine populations (Cowen & Sponaugle, 2009).

1.4: Detection of possible barriers to the gene flow throughout the inhabited area

According to the actual circulation patterns, several marine fronts exist within the distribution range of the black goby, which can act as a barrier to the gene flow for some marine organisms. Probably the most intensively studied is the semi-permeable Almeria-Oran front, which acts as a phylogeographical break for many marine organisms, separating populations/species in the Atlantic Ocean and the Mediterranean Sea, whereas populations of other species with similar life-history patterns do not show any genetic differences on the both sides of the front (Patarnello et al., 2007).

Another front reducing the gene flow of many marine organisms in the western Mediterranean basin is the Balearic front. An important characteristic of this front is the seasonality, as the density gradient reduces at the autumn (Barral et al., 2021), which enables the late-spawning organisms to cross this front (Galarza, Carreras-carbonell, et al., 2009). The connection of the western and eastern basins through the Sicilo-Tunisian Channel can also act as a barrier, mainly for the lagoon and shallow water inhabitants who underwent habitat loss during the periods of glaciation leading to the bottlenecks followed by genetic differentiation of separated populations (Mejri et al., 2009).

From the eastern basin, probably the most studied is the biogeography of the Adriatic subbasin. The glacial climatic oscillations (Dumitru et al., 2021) together with the circulation patterns led to the isolation of the populations of some marine fish species in the north Adriatic (Stefanni & Thorley, 2003; Sefc et al., 2020).

In general, the function and importance of the proposed phylogeographic fronts within the Mediterranean Sea still remains understudied (Pascual et al., 2017). The last important biogeographic barrier is the narrow Turkish strait system, separating the Black Sea basin from the Mediterranean Sea, which prevents population connectivity of many marine organisms (see 1.2.1).

1.5: The phylogeography of gobies of the European coast

The order Gobiiformes is one of the richest orders of the teleost fishes, presenting enormous variety of biological and ecological strategies in the both marine and freshwater environment. (Patzner et al., 2011). In the European fresh water and marine habitats, this order is represented mainly by the rich family Gobiidae (*sensu* Agorreta et al. 2013), with more than 90 species of gobies described so far. The phylogenetic study of Agorreta et al. 2013 has divided the family into the three evolutionary lineages, the *Pomatoschistus*-, *Aphia*- and *Gobius*- lineage. The present work is focused on the black goby (*Gobius niger*), a common benthic goby (Fries et al., 1892) inhabiting the shallow coastal waters of Europe and north Africa (see Fig. 1)(Miller, 1986), which belongs into the *Gobius*- lineage *sensu* Agorreta et al. (2013).

The phylogeography research was conducted mainly on the species of the sand gobies, particularly on the paraphyletic (Thacker et al., 2019) genus *Pomatoschistus*, the *P. marmoratus* (Riso, 1810) (Mejri et al., 2011; Seyhan-Ozturk & Engin, 2021), *P. tortonesei* Miller, 1969 (Mejri et al., 2009), *P. minutus* Pallas, 1770 (Stefanni & Thorley, 2003; Gysels et al., 2004; Emilie Boissin et al., 2011) and *P. microps* Krøyer, 1838 (Tougard et al., 2014) belonging into the “gobionelline-like” lineage of the family Gobiidae (Agorreta et al., 2013).

The gobies from the genera *Pomatoschistus* usually occupy shallow coastal habitats, mainly lagoons and protected bays with the soft sediments. They present the similar sedentary lifestyle and mating tactic as *Gobius niger* (Bouchereau & Guelorget, 1998; Christophe Pampoulie et al., 1999), but much longer planktonic larval duration of 40 – 50 days in laboratory conditions (Locatello et al., 2017). The study on *P. minutus* has described the complex phylogeographic structure of the species and revealed the significant evolutionary unit in the northern Adriatic Sea (Stefanni & Thorley, 2003). The integrity of the *P. minutus* species has been challenged by (Boissin et al., 2011), who proposed rather to distinguish the Atlantic, Mediterranean and the Adriatic populations as a distinct species, which is supported also by the results of Tougard et al. (2021).

The separation of the western and eastern population divided by the Sicilo-Tunisian Strait has been described for *Pomatoschistus tortonesei* (Mejri et al., 2009), and the similar pattern revealed for *Pomatoschistus marmoratus* (Mejri et al., 2011). Moreover, the population of *P. marmoratus* shows marked structure in the central and eastern Mediterranean area (Mejri et al., 2011; Seyhan-Ozturk & Engin, 2021).

From the "gobiine-like" clade of the family Gobiidae (Agorreta et al., 2013), the phylogeography has been studied only for the species *Gobius niger* and *Aphia minuta* Risso, 1810 in the small-scale study of Giovannotti et al. (2009) and for *Gobius geniporus* Valenciennes, 1837 and *Gobius cruentatus* Gmelin, 1789 (Čekovská et al., 2020). Giovannotti et al. (2009) has focused mainly on the life-history difference between the *G. niger* and *A. minuta* relating it with the phylogeographic patterns. The *G. niger* individuals collected from the Adriatic Sea showed clear segregation from the western Mediterranean, whereas the population of *A. minuta* was only weakly structured.

In contrast with *G. niger* and *A. minuta*, (Giovannotti et al., 2009), the two species *Gobius geniporus* and *Gobius cruentatus* does not show any geographically structured populational structure (Čekovská et al., 2020), even though they have analogous life-history patterns with *G. niger*. The origin of the black goby lineage has been estimated 17,75 million years ago (Thacker, 2015) when the common ancestor lineage split into *Gobius niger* and *Zosterisessor ophiocephalus* Pallas, 1814 species (Agorreta et al., 2013; Thacker, 2015). Given the proposed age of the *G. niger* lineage, the evolution of the population of the *Gobius niger* was shaped by the geological and climatic processes affecting the region of the Mediterranean Sea and the North-eastern Atlantic Ocean.

1.6: Biology and ecology of the *Gobius niger*

1.6.1: Description of the species

Gobius niger is a small benthic fish. Although the maximum documented total length for the species is 16.5 cm (Fabi & Giannetti, 1985) they usually do not exceed 15 cm (Filiz & Toğulga, 2009). The body is flattened dorsoventrally in the anterior part and laterally in posterior part. The colour is pale brown, with lateral dark blotches and spots. The sexual dimorphism is present, males being generally bigger than females and living longer (Filiz & Toğulga, 2009). Males are darker than females and they can show blue-opalescent colour. Also, the fourth ray of the dorsal fin of male individuals is prolonged (Fries et al., 1892; Miller, 1986; Quispe-Becerra, 2014). They can be distinguished from closely related species by the distribution of the cephalic pores, which can be seen on the Fig. 4. Miller (1986) has described some differences between Mediterranean and Atlantic populations (earlier

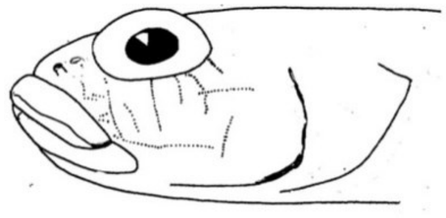

Figure 4.: Distribution of the cephalic pores (Kara & Quignard, 2019)

recognised as two different species, *G. niger* and *G. jozo* Linnaeus, 1758). The Atlantic "*G. niger*" should have incomplete scale cover on the nape, with smaller scales than those present in the Mediterranean individuals. In comparison, the Mediterranean "*G. jozo*" presented complete imbricate scale cover on the nape and the bigger scales than the previous ones (Miller, 1986). Today, the division of the species into several sub-species or even different species is not accepted (Fives, 1970; Froese & Pauly, 2022).

1.6.2: Distribution and ecological valence

Individuals of this species are restricted to the shallow coastal areas, usually no deeper than 50 meters (Nieto & Alberto, 1993; Boban et al., 2013). In the northern parts of distribution area, offshore migration of the black gobies to deeper waters was documented during the winter, reaching the depth of 200 meters (Fries et al., 1892; Nash, 1984). According to Bouchereau & Guelorget (1997), the black goby has a migratory potential in the Atlantic and in the Adriatic Sea, responding to local environmental conditions, but without any general migratory pattern.

The temperature limits were experimentally defined by Paris & Quignard (1971) in the Thau lagoon (France, western Mediterranean) as 4°C (lower limit) and 35°C (upper limit).

The black goby inhabits shallow areas, including lagoons, bays, ports or estuaries, inhabiting also brackish waters with salinity no lower than 3.5‰ (Paris & Quignard, 1971), but it usually occurs in salinities no lower than 6‰ (Vaas et al., 1975). Pampoulie et al., (1999) has proposed to call them apmhihaline, due to the reported difference in salinity preferences among larvae, juveniles and adults and supposed migration in lagoons, but the salinity in some lagoons and estuaries can vary seasonally, depending on the freshwater input and evaporation, as for example in the case of Aveiro lagoon (Portugal, North-east Atlantic) (Arruda et al., 1993). In some lagoons with anthropogenically closed openings, which had a consequence in stabilisation of the salinity regime and prevention of fish migration, the population of *Gobius niger* has become dominant after closure (Vaas et al., 1975; Doornbos & Twisk, 1987).

1.6.3: Habitat

The black goby can be found on soft sediments, mostly on the sand or mud (Boban et al., 2013; Kara & Quignard, 2019), with presence of stones, shells (Iglesias, 1981) or small rocks outcrop from the bottom (Vesey & Langford, 1985), which they may use like a nest protection. A preference of juveniles and adults for the seagrass (Malavasi et al., 2005) or algae cover (Vaas et al., 1975) was described (Casabianca & Kiener, 1969; Magnhagen, 1988; Malavasi et al., 2005), whereas in Novigrad sea, Kruschel & Schultz (2011) reported a switch of habitat preference between juveniles and adults as a response to a predator (*Zosterisessor* *ophiocephallus*, another gobiid occupying the seagrass) presence. In that case, juveniles usually prefer the bare sediments neighbouring seagrass, rather than seagrass meadows. In Versee Meer (Netherlands, North Sea), they can be found in the cover of *Chaetomorpha* Kützing, 1845, *Ulva* Linnaeus, 1753 or *Callithamnion* Lyngbye, 1819 (Vaas et al., 1975). In Mar Menor (Spain, Western Mediterranean), the black goby has become a dominant species in the newly introduced cover of *Caulerpa prolifera* (Forsskål) J.V. Lamouroux, 1809, which appeared after human induced degradation of sandy bottom shifting to the mud covered with *Caulerpa* (Pérez-Ruzafa et al., 2006). In Óbidos lagoon (Portugal, North-east Atlantic), they can be found on both sandy and muddy substrates, with or without any vegetation cover, but with a strong positive effect of vegetation (*Ulva* and eelgrass) on the population density (Serrano Gordo & Nogueira Cabral, 2001). Importance of the macrophytes for the presence of the black goby is discussed also by Fries et al. (1892) and Pampoulie et al. (1999, 2001). In contrast, no vegetation cover as a habitat of the black goby was described by (Vesey & Langford, 1985) from the Stanswood bay (Great Britain, North-east Atlantic). In the Mediterranean Gulf of Fos (France, Western Mediterranean), the Black goby prefers muddy substrates with only scarce presence of developed macroalgae (Letourneur et al., 2001).

1.6.4: Feeding ecology

The black goby is a demersal opportunistic predator (Vaas et al., 1975; Labropoulou & Markakis, 1998; Hajji et al., 2013). Kruschel & Schultz (2011) described his strategy as a “sit-and-pursue or wait-and-chase predator”. Upon detection of prey, including smaller individuals of its own species, *G. niger* “follows it across the sediment in a chase-like manner that can last several minutes and results in repeated prey attacks”. According to Labropoulou & Papadopoulou-Smith (1999), they black gobies are detecting prey mainly through visual and olfactory cues, and thus they are not capable to detect burrowed organisms.

The most important prey usually consists of polychaetes, crustaceans (mainly decapods, with a smaller portion of amphipods and cumaceans), and molluscs (Vaas et al., 1975; Bell & Harmelin-Vivien, 1983; Labropoulou & Markakis, 1998; Labropoulou & Papadopoulou-Smith, 1999) , but the proportion of these groups as a prey can be highly variable between regions. The consumption of foraminifera was also mentioned by some authors (Bell & Harmelin-Vivien, 1983; Casabianca & Kiener, 1969; Filiz & Toğulga, 2009), but it is proposed to be ingested accidentally (Filiz & Toğulga, 2009). Teleosts (including its own species) were found only occasionally in the samples from Aegean Sea (Filiz & Toğulga, 2009; Filiz, 2013) and from Corsica (Casabianca & Kiener, 1969), but they were found to be dominant prey (60 – 92% of wet mass) in the Mauguio lagoon (France, Western Mediterranean) (Joyeux, Tomasini, et al., 1991). Hajji et al. (2013) described the presence of teleost in the stomach of older fishes, and he postulates that difference in the diet between regions can be result of different prey availability and opportunism. In the older publications also consumption of some algae was documented, which are probably ingested accidentally during the hunting of amphipods (Casabianca & Kiener, 1969; Fries et al., 1892). Vesey & Langford (1985), Joyeux et al. (1991), and later Hajji et al. (2013) also described seasonal changes in the diet, as the proportion of molluscs, crustaceans and teleosts vary seasonally during the year. Vesey & Langford (1985) explained this shift as an effect of different mobility capacities of the black gobies under changing environmental conditions during the year, whereas Hajji et al. (2013) suggested that the difference can be due to the changing prey availability during the year. Also the ontogenetic shift in the prey composition was pointed by Vaas et al. (1975), who noticed that younger individuals (smaller than 6 cm) do not consume fish prey, and also they do consume less molluscs, contrary to individuals bigger than 6 cm. This phenomenon was also noticed by Hajji et al. (2013). According to the trophic level, *G. niger* belongs into the ecological group of "omnivores, with a preference for animals" (Stergiou & Karpouzi, 2002), and this position was confirmed by Hajji et al. (2013). The trophic level of this species lays approximately between 3.2 and 3.56 (Hajji et al., 2013), but this number can vary seasonally, and thus they can be recognised as both secondary or tertiary consumers, depending on the part of the year (Como et al., 2018).

1.6.5: Reproduction and ontogenesis

Most individuals become adults during their first (Filiz & Toğulga, 2009; Rasotto & Mazzoldi, 2002) or second year of life (Vesey & Langford, 1985). The spawning season begins during the spring, and various authors suggest that it is induced by the increasing water temperature (Vaas et al., 1975; Filiz & Toğulga, 2009; Hajji et al., 2013), so the beginning and the duration of the spawning can vary regionally (Bilgin & Onay, 2020) (see Tab. 1). Vaas et al. (1975) has reported that the 12°C is the limit for beginning of spawning period in the Veerse Meer (Netherlands, North Sea). Nash (1984) did not confirm any exact temperature limit for spawning in the case of population from Oslofjorden (Norway, North Sea), although the water temperature was rising rapidly at the beginning of spawning period. The idea of the temperature control of the beginning of reproduction was also supported by Filiz & Toğulga (2009).

The reproductive strategy of the black goby was described as a protracted iteroparity (Giovannotti et al., 2009), as both sexes can reproduce repeatedly for several consecutive years. The males of the black goby build nests under the stones, shells, or another hard substrate on

Table 1: Comparison of the spawning periods for *G. niger* in different areas (taken from Bilgin et al., 2020)

Spawning months												Latitude	Study area	Reference
J	**F**	**M**	**A**	**M**	**J**	**J**	**A**	**S**	**O**	**N**	**D**			
			●	●	●							59°N	North Sea, Oslofjorden, Norway	Nash, 1984
				●	●	●	●					51°N	North Sea, Veerse Meer, Netherlands	Vaas et al., 1975
			●	●	●	●						40°N	Atlantic, Aveiro lag., Portugal	Arruda et al., 1993
		●	●	●	●	●	●	●				38°N	Atlantic, Portugal	Silva &Gordo, 1997
		●	●	●	●	●	●	●				43°N	Mediterranean, Mauguio lag., France	Joyeux et al., 1991
		●	●	●	●							34°N	Mediterranean, gulf of Gabès, Tunisia	Hajji et al., 2013
			●	●	●	●	●					43°N	Adriatic Sea, north of Ancona, Italy	Fabi &Giannetti, 1983
			●	●	●	●	●	●				45°N	Adriatic Sea, Venetian lag., Italy	Immler et al., 2004
		●	●	●	●	●	●	●				38°N	Aegean Sea, Izmir Bay, Turkey	Özaydın et al., 2007
		●	●	●	●							38°N	Aegean Sea, Izmir Bay, Turkey	Kınacıgil, 2008
		●	●	●	●	●	●	●	●			38°N	Aegean Sea, Izmir Bay, Turkey	Filiz & Toğulga, 2009
		●	●	●	●	●						40°N	Marmara Sea, All coasts, Turkey	Kırdar & İşmen, 2018
			●	●	●	●	●					41°N	Black Sea, Rize coast, Turkey	Bilgin et al., 2020

the bottom (Fries et al., 1892; Vaas et al., 1975; Filiz & Toğulga, 2009). The females place their eggs inside the nest, where they are fertilised and protected by males afterwards. One female can potentially produce 54 400 eggs in the first year and up to the 155 900 eggs in the second year of life (Joyeux et al., 1991) and in one nest, up to 20 000 and 80 000 can be present, depending on the nest surface (Mazzoldi & Rasotto, 2002). At least three different batches of eggs of different females were observed in the nest, so the *G. niger* can be reckoned as a polygynous species (Mazzoldi & Rasotto, 2002).

The reproductive strategy of males can change during the ontogenesis, depending on the availability of proper substrate for the nest (Immler et al., 2004). If there is a lack of suitable nesting sites, the youngest males present sneaking behaviour. The sneakers do not build nest, they mimetise females, trying to enter nests of parental males to fertilise eggs laying there (Mazzoldi & Rasotto, 2002). The sneaker males lack the black colouration and the prolongation of the fourth ray of the first dorsal fin (Rasotto & Mazzoldi, 2002), and their ejaculate composition differs compared to the dominant males. They produce higher amount of more mobile sperm than the parental males, which invest more energy to the nest building and protection (Rasotto & Mazzoldi, 2002; Scaggiante et al., 2005). The parental individuals protect the clutch for eight to nine days and after that, they move out to establish new nest. One male can build up to five nests during one spawning season (Mazzoldi & Rasotto, 2002).

The larvae hatch after 20 days of incubation (Vaas et al., 1975). The newly hatched larvae measure approximately 2.5 mm and have a specific pigment colouration on the ventral and dorsal parts of the body (Petersen, 1919, Vaas, 1975). According to Planes (1998), the average larval stage duration is 28 days. Opposite to the adults, which seems to be strictly demersal, the larval phase takes place in the open sea as a part of the zooplankton, thus providing dispersal abilities and potential to colonize new localities (Giovannotti et al., 2009; Vaas et al., 1975). The larvae become sedentary when they reach the size of 12 mm (Fives, 1970).

The post-larval growth was widely discussed by Filiz & Toğulga (2009) and more recently by Kara & Quignard (2019). Bouchereau & Guelorget (1997) have remarked that the growth characteristics differ among Atlantic and Mediterranean populations. Based on that study, the Atlantic populations should show more "protracted" life cycle, with a steady growth rate, whereas the Mediterranean populations have a "contracted" life cycle characteristics, with a higher growth rate during the first year of life. This pattern has been described also for the populations of *Pomatoschistus minutus* and *Pomatoschistus microps*, and it was supposed to be

Table 2: Comparison of maximum age and size at age records, (taken from Filiz *et al.*, 2009)

Area	**Locality**	**Sex**	**0+**	**I**	**II**	**III**	**IV**	**V**	**References**
	Adriatic Sea	♂	7.7	9.4	11.9	13.5	14.5	15.5	Fabi &Giannetti, 1985
		♀	6.2	7.8	9.5	10.4	11.8		
	Mauguio lag., W. Mediterranean	♂	-	8.8-9.6	9.6-12.0	12.0-13.2	13.6	-	Joyeux *et al.* 1991
		♀	-	8.4-9.2	9.2-11.6	11.6-12.4	-	-	
	Venetian lag., Adriatic Sea	♂	-	7.4	10.1	12.2	12.8	-	Rasotto & Mazzoldi, 2002
	Izmir Bay, E. Mediterranean	♂	8.18	10.34	11.93	13.29	14.14	14.78	Filiz *et al.* 2009
		♀	6.67	8.10	9.90	11.33	12.26		
		♂+♀	7.76	9.76	11.52	12.96	13.81	14.78	
	Verse Meer, North Sea	♂	5.5	8.2	9.5	12.0			Vaas et al., 1975
		♀	5.5	8.1	9.6	10.5	11.1		
	Norwegian coasts, North Sea	♂+♀	-	4.4	7.1	8.6	9.6	9.3	Nash, 1984
	Stanswood Bay, NE Atlantic	♂+♀	~3	5.6	9.0	10.9	-	-	Vesey & Langford, 1985
	Grevelingen Lake	♂+♀	4.7	8.0-8.5	12.2-12.5	-	-	-	Doornbos & Twisk, 1987
	Ria de Aveiro, NE Atlantic	♂	7.6	10.8	11.8	-	-	-	Arruda et al., 1993
		♀	7.2	10.5	11.5	-	-	-	
	Obidos lag., NE Atlantic	♂	7.8	10.5	12.2	13.5	-	-	Silva & Gordo, 1997
		♀	8.0	10.3	11.9	12.0	-	-	

result of different temperature of the water and differences in the day length between Mediterranean and North east Atlantic localities. Comparing different studies, Filiz (2009) described the main differences among Atlantic and Mediterranean populations (see the Table 2). The Mediterranean individuals can reach bigger lengths than the individuals from Atlantic coast. Also, the Mediterranean populations show longer life span, the individuals of the age V were observed (based on the direct otoliths observations), which was described only once in Atlantic region (see Tab. 2). The differences in sampling methods and characteristics of the sampled locations are supposed to have possible influence on the results (Filiz & Toğulga, 2009).

2: Aims of the thesis

The aims of the thesis are following:

1) to examine the genetic diversity of populations of the black goby *Gobius niger*

2) to elucidate whether the genetic structure reflects geography, and to try to disentangle possible migration barriers, according to the biology and ecology of the species in the relation to the hydrologic patterns across the distribution area.

3: Material and methods

3.1.: Sample collection

The samples for the study were collected by our team or by our collaborators from the 28 localities (see Fig. 5) The pieces of the tissue from the right pectoral fin were stored in 96% ethanol. The list of individuals per locality with appropriate coordinates is summarised in Table 3.

Table 3: Sampling sites with corresponding coordinates with number of sequences of each genetic marker obtained per locality. The samples from localities marked with indices were merged together for analyses.

No.	Sea basin	Country	Locality	Coordinates	N_{CytB}	N_{dloop}
1	North Sea	Norway	Egersund	58°26'52.5"N 5°59'11.8"E	20	26
2	North Sea	Norway	Kristiansand	58°08'15.5"N 8°01'24.2"E	17	26
3	North Sea	Norway	Oslofjord	59°29'02.8"N 10°33'15.4"E	26	28
4	North Sea	Sweden	Gullmarn	58°21'25.3"N 11°35'06.2"E	9	26
5	English Channel	United Kingdom	Pilsey Island	50°48'02.9"N 0°54'34.3"W	27	28
6[1]	Bay of Biscay	France	Crozon	48°14'03.4"N 4°29'10.8"W	4	5
7[1]	Bay of Biscay	France	Concarneau	47°52'02.9"N 3°55'05.1"W	27	26
8	Atlantic Ocean	Spain	Galicia	42°55'29.2"N 9°10'28.6"W	22	30
9	Atlantic Ocean	Portugal	Arrábida	38°26'27.1"N 9°06'31.4"W	27	33
10	Atlantic Ocean	Portugal	Troia peninsula	38°29'07.2"N 8°53'16.2"W	11	26
11	Atlantic Ocean	Portugal	Ria formosa	37° 0'10.42"N 7°59'3.77"W	36	39
12	Bay of Cadiz	Spain	Cádiz	36°31'01.7"N 6°12'01.8"W	9	11
13	Atlantic Ocean	Spain	Tenerife	28°30'18.0"N 16°11'17.0"W	29	22
14	Gulf of Lion	France	Banyuls	42°29'40.0"N 3°09'06.4"E	18	23
15	Gulf of Lion	France	Berre lagoon	43°26′45″N 5°06′50″E	7	7
16	Western Mediterranean	Algeria	Annaba	36°55'02.1"N 7°46'12.4"E	23	35
17	Northern Adriatic Sea	Croatia	Soline, Krk	45°09'07.6"N 14°36'05.9"E	29	29
18[2]	Southern Adriatic Sea	Montenegro	Strp	42°30'14.4"N 18°40'10.0"E	5	5
19[2]	Southern Adriatic Sea	Montenegro	Kostanjica	42°29'06.5"N 18°40'13.3"E	27	27

20	Southern Adriatic Sea	Albania	Vilunit Velipoje	41°52'30.9"N 19°25'57.0"E	3	3
21[3]	Ionian Sea	Greece	Syvota	39°23'59.3"N 20°14'04.8"E	25	25
22[3]	Ionian Sea	Greece	Moraitika, Korfu Island	39°28'57.0"N 19°56'06.0"E	2	3
23	Ionian Sea	Greece	Petalidi	36°57'32.9"N 21°56'05.9"E	6	6
24[4]	Aegean Sea	Greece	Paralia Rachon	38°52'34.1"N 22°47'08.5"E	17	20
25[4]	Aegean Sea	Greece	Kastari, Euboia Island	37°58'22.9"N 24°32'15.6"E	1	1
26[4]	Aegean Sea	Greece	Petali Islands	38°00'53.0"N 24°16'51.0"E	1	1
27	Black Sea	Ukraine	Odessa	46°28'27.4"N 30°46'04.3"E	5	4
28	Black Sea	Bulgaria	Varnensko Lake	43°11'47.4"N 27°48'24.5"E	17	20
				Total no. of individuals:	**450**	**535**

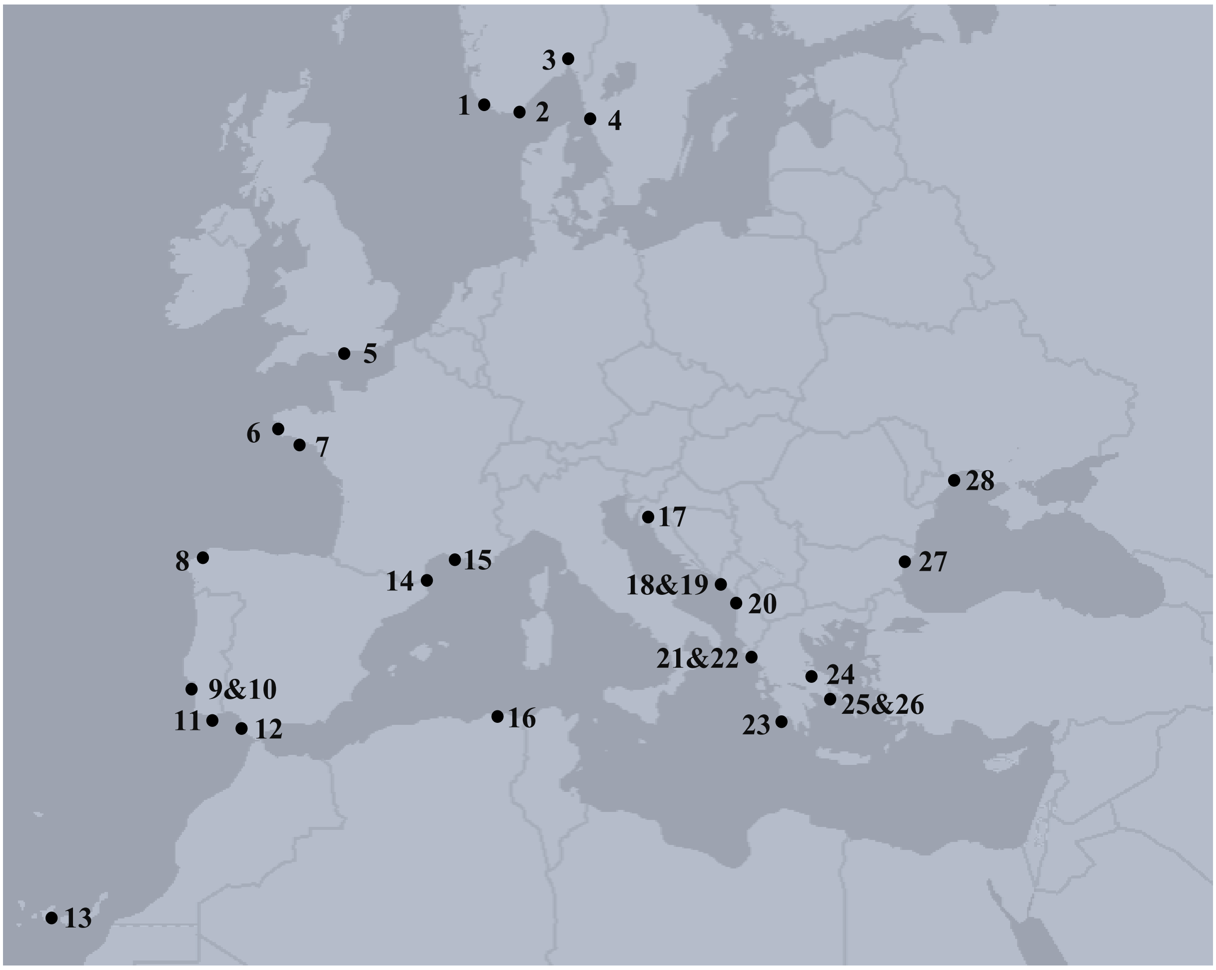

Figure 5: Map showing the distribution of localities corresponding to the Tab. 3.

3:2.: DNA isolation

To extract the DNA from the fin tissue, the *Geneaid® DNA Isolation Kit* has been used. The protocol of Manufacturer was followed, with a minor modification. The temperature and the time for tissue dilution has been optimized, from the incubation at 60°C for 30 minutes proposed by the Manufacturer to 56°C incubation lasting at least 3 hours, which has led to the better tissue digestion. At the end of the isolation, the elution was divided into two separated steps. The first elution was done with 100 µl of elution buffer, and it has been incubated for the 10 minutes in the room temperature. The centrifugation (14 000 RPM) of the first eluate was immediately followed by the second elution, which was performed with 50 µl of elution buffer, incubated again for the 10 minutes in the room temperature. The DNA isolates were stored at - 20°C.

3.3.: PCR amplification

For the purpose of this study, two mitochondrial genes, Cytochrome b and the D-loop (part of the control region) were amplified.

The mitochondrial genome is ideal for the study of phylogeography due to the steady and fast mutation rate and maternal inheritance reducing the effective population size, which enables to find phylogenetic relationships even on the scale of subpopulations (Wilson et al., 1985; Rüber & Agorreta, 2012).

Cytochrome b is a mitochondrial gene coding one of the eleven subunits of the mitochondrial respiratory chain complex III (Massie et al., 2010). The cytochrome b has a crucial role in the correct assembly and function of the complex (di Rago et al., 1993) and thus the variability of the sequences can be found mainly on the 3rd position of codon (Habib et al., 2011). In this study, the entire cytochrome b (1140 base pairs) of 450 individuals of *Gobius niger* was amplified. In the case of insufficient quality of the part of the material, the amplification of the cytochrome b had to be divided into two parts using the combination of internal (RoulF, GniroR) and external (GluF, ThrR) primers (Machordom & Doadrio, 2001).

The three-strand displacement loop, so called D loop, is a part of the control region of the mitochondrial DNA with reduced functional constrains, leading to faster evolution of the segment (Lee et al., 1995; Baker & Marshall, 1997). The smaller size of the sequences enhances the possibility to amplify the D loop even from the material damaged by the inappropriate storage of the samples and also it reduces price of the sequencing. For the purpose of this thesis, the 296 base pairs long segment of control region of 535 individuals was amplified using the primers Lpro1 and H-DL1 (Ostellari et al., 1996). The sequences of all primers are shown in Tab. 7.

For the amplification of the selected parts of mitochondrial DNA by the polymerase chain reaction (PCR), the mixture of the total volume of 14 µl for the both cytochrome b and for the D loop was prepared with the use of the PPP master mix (Top-Bio). The composition of the mixture is described in detail in the Tab. 4. The PCR was performed in the Bioer Gene Touch^{TM} and Proflex^{TM} PCR System thermocyclers using the protocols described in the Tab. 5 for the D loop and the Tab. 6 for the Cytochrome b, respectively.

Table 4: The protocol for the preparation of PCR mix

Composition of the PCR mixture	***Volume 1 PCR reaction***
PPP master mix	7.4 µl
PCR ultrapure H_2O	3.8 µl
Forward primer	0.4 µl
Reverse primer	0.4 µl
DNA isolate	2 µl
Total volume	**14 µl**

Table 5: The protocol for the D loop PCR amplification

Reaction	**Temperature**	**Time**	
Initial denaturation	94 °C	7 min	
Denaturation	94 °C	30 s	35x
Annealing	55 °C	30 s	
Elongation	72 °C	1 min	
Final Elongation	72 °C	7 min	
Hold	4 °C	∞	

Table 6: The protocol for the Cytochrome b PCR amplification

Reaction	**Temperature**	**Time**	
Initial denaturation	94 °C0	3 min	
Denaturation	94 °C	45 s	40x
Annealing	46 °C	1 min 30 s	
Elongation	72 °C	1 min 45 s	
Final Elongation	72 °C	7 min	
Hold	4 °C	∞	

Table 7: The sequences of primers used for PCR and for sequencing for Cytochrome b and for D loop

Gene	Primer	Direction	Sequence 5' → 3'	Reaction
Cytochrome b	GluF	forward	AAC CAC CGT TGT ATT CAA CTA CAA	PCR
	GniroR	reverse	GGT CCT TGT AGG ARA AGT	PCR, sequencing
	RoulF	forward	GCA ACM GTK ATT ACS AA	PCR, sequencing
	ThrR	reverse	ACC TCC GAT CTT CGG ATT ACA AGA CCG	PCR
D loop	L-pro1	forward	ACT CTC ACC CCT AGC TCC CAA AG	PCR, sequencing
	H-DL1	reverse	CTG AAG TAG GAA CCA GAT GCC AG	PCR

3.4.: Electrophoresis

The horizontal electrophoresis was performed for the evaluation of the length and quality of the PCR products. The gel was prepared by dilution of 75 g of agarose in Tris/borate (TBE) buffer (Voytas, 2000), with addition of the fluorescent dye *GelRed® Nucleic Acid Gel Stain* in the concentration proposed by the Manufacturer. Once the gel has become solid, the 2 µl of PCR product were injected into the hollows. For each electrophoresis, the 1 µl of the ladder (GeneRuler 100 bp Plus DNA Ladder) was used as a reference of the PCR product length. The electrophoresis was set to 150 V for 30 minutes and the results were checked under the UV light.

3.5.: Purification

The purification of the PCR products was realized with the *ExoSAP-IT® PCR Product Cleanup Reagent*. The ExoSAP-IT removes the excess primers and unincorporated nucleotides by enzymatic hydrolyzation. The manufacturer´s protocol was modified reducing the volume of the ExoSAP-IT reagent need for the purification and thus reducing the price of the procedure. The ExoSAP-IT reagent was diluted 10x in the PCR water, and then the 5 µl if the PCR product was mixed with 2 µl of the diluted ExoSAP-IT. After mixing, the samples were incubated at 37°C for 60 minutes which has led to degradation of remaining primers and nucleotides, followed by the incubation at 80°C for 15 minutes inactivating the ExoSAP-IT reagent.

3.6.: Data analysis

The Sanger sequencing of PCR products was performed by the Macrogen Europe (https://www.macrogen-europe.com/). Due to the low quality of the part of the material, the cytochrome b sequences were divided in two parts and they were sequenced separately with corresponding internal primers (Gniro R and RoulF).

The DNA sequences of D loop and cytochrome b were inspected manually in *Chromas v2.6.6.* (http://technelysium.com.au/wp/chromas/). The sequences of cytochrome b were assembled manually in *BioEdit v7.2.5* (Hall, 1999). The alignment of complete cytochrome b sequences and D loop sequences and the cut of the primer sequences was performed in *BioEdit* too.

The conversion of the alignment from FASTA to NEXUS format was performed in the online Alignment Transformation EnviRonment (http://www.sing-group.org/ALTER/, (Glez-Peña et al., 2010)). The genetic variability was assessed by *DNA Sequence Polymorphism v6.12.03 x64* (Rozas et al., 2017). The number of haplotypes, haplotype diversity and nucleotide diversity were computed. Moreover, the Fu & Li´s (Fu & Li, 1993) and Tajima´s (Tajima, 1989) tests of neutrality were performed to observe the demographic history of the population. The diversity measures and the test of neutrality were computed for entire population (for both the cytochrome b and the D loop) and also separately for each geographic sub-unit.

The haplotype networks were computed in the program *TCS v1.23.2* (Clement et al., 2000), applying statistical parsimony. The connection limit for the computation was set on 95%. The generated haplotype networks were graphically edited in online graphic editor *tcsBU* (Santos et al., 2016).

For all the analyses of the dataset, apart from the haplotype network reconstruction and the Mantel test, some of the localities with a small number of individuals were grouped with a nearby localities, in the case that the union make biological and geographical sense and the sequences did not present important difference in the haplotype network. Specifically, the localities grouped in the analyses together are: Crozon (no. 6) with Concarneau (no. 7); Kastari (no. 25) with Petali (no. 26) and with Paralia Rachon (no. 24); Syvota (no. 21) with Moraitika (no. 22) and Strp (no. 18) with Kostanjica (no. 19), see Tab. 3.

The software *MEGA v11* (Tamura *et al.* 2021) was used to compute the genetic distances between pairs of sequences and between and within chosen groups of sequences, applying the uncorrected p-distance method. For the global population, the p-distances of both cytochrome b and D loop were computed between and within particular haplotype groups as divided in the haplotype networks, and all the sequences from each haplotype group were included, even if

Table 8: Division of the studied area into the regions and subregions. Corresponding localities are numbered as in the Tab. 3

Region	Subregion	Localities
Atlantic Ocean and the North Sea	North Sea	**1 - 4**
	English Channel &Bay of Biscay	**5 - 7**
	Iberian basin	**8 - 12**
	Canary Islands	**13**
Western Mediterranean	northern western-Mediterranean	**14 - 15**
	southern western Mediterranean	**16**
North Adriatic Sea	North Adriatic Sea	**17**
Central Mediterranean	Southern Adriatic Sea	**18 - 20**
	Ionian Sea	**21 - 23**
	Aegean Sea	**24 - 26**
Black Sea	Varnensko Lake	**27**
	Odessa Bay	**28**

they were from geographically distant localities. To reveal genetic structure on the regional scale, the p-distances were computed also between and within particular geographic units as described in the Tab. 8. Moreover, in the western Mediterranean and in the northern Adriatic regions, where more than one haplotype group was detected, the p-distances were also computed between and within the haplotype groups.

The genetic structure of the population was evaluated in *Arlequin v3.5.2.2* (Excoffier, 2015). The AMOVA is structured into two levels, the first level ("populations") corresponds to the localities, while the second level ("groups") correspond to the regions or subregions as in the tab. 8. In *Arlequin*, the analysis of molecular variance (AMOVA) and the calculation of fixation indices (F_{ST}) were performed, both with 15 000 permutations. For assessing statistical significance of pairwise F_{STs}, the Bonferroni correction was applied. For the global population, the division was made according to the regions. The first analysis was performed on higher scale, comparing the populations from the Atlantic Ocean and western Mediterranean, northern Adriatic, Black Sea and the Central Mediterranean. Subsequent analyses focused on the finer scale within the regions, and the second level of AMOVA was set to subregions.

Evaluation of the importance of geographic distance on the differentiation of the population was performed in *Excel v16.0.15831.20208* (Microsoft, 2018) using the GenAlEx 6.5 (Peakall & Smouse, 2006, 2012) package to compute Mantel test of isolation by distance

(Mantel, 1967). The shortest sea distance among pairs of localities was measured by hand in online map program Mapy.cz (https://www.mapy.cz, © Seznam.cz, a.s.). The Mantel test was calculated with 999 permutations.

To select the best-fit model of nucleotide substitution, the *jModelTest v2.1.10* (Darriba et al., 2012, Guindon & Gascuel 2003) program was used, applying the Akaike and Bayesian information criteria (AIC and BIC). The model test prioritized different models according to the criteria, so the most appropriate model had to be chosen according to the order given by both criteria.

The maximum likelihood phylogenetic trees were constructed using the Science Gateway portal CIPRES (Miller et al., 2010). The maximum likelihood approach was assessed in Randomized Axelerated Maximum Likelihood tool (RAxML-HPC2 8.2.12, Stamatakis, 2014), using the model GTR+G. She supports of the tree nodes were estimated by applying 1000 nonparametric bootstrap replicates.

Estimation of past population dynamics and fluctuation of the effective population size was conducted under Bayesian coalescent method in the program *BEAST v1.10.4* (Suchard et al., 2018). To prepare the BEAST XML file, the program *BEAUti v1.10.4* was used. The substitution model (GTR) and the site heterogeneity model (Gamma + Invariant Sites) were selected according to the *jModelTest v2.1.10* (Darriba *et al.* 2012, Guindon & Gascuel 2003) with estimated base frequencies. As a tree prior, the coalescent Bayesian skyline prior was set, with the default number of groups. To achieve a reasonable effective sample size, the length of chain was set on the 450 000 000 and the computation was repeated three times independently, and the runs were combined using *LogCombiner v1.10.*, with the burn-in set to 45 million iterations per each run. The files generated by the Bayesian MCMC runs performes in *BEAST v1.10.4* were analysed in the program *Tracer v1.7* (Rambaut et al., 2018). For the lack of the dating point, the exact dating of the dynamics was not performed and the x-axis displays the time in mutation units per nucleotide position.

4: Results

4.1.: Cytochrome B

A total of 450 sequences of the cytochrome b of *Gobius niger*, with the length of 1140 base pairs were analysed. The very high haplotype diversity (Hd = 0.9760) and also high nucleotide diversity ($\pi = 0.02438$) indicate the complexity of the population (see the Tab. 9). The diversity measures per locality are in the Tab. 9. The Fu & Li´s tests of neutrality had significant negative values, whereas the Tajima´s D test of neutrality was also negative, but not significant ($p > 0.10$). The singletons are in excess against neutrality model, which is in accordance with high nucleotide diversity.

Table 9: Diversity measures of cytochrome b of *G. niger*. Ns – number of sequences, S – number of segregating sites, Nh – number of haplotypes, Hd – haplotype diversity, π – nucleotide diversity, Fu&Li´s D, Fu&Li´s F and Tajima´s D. Statistical significance of Fu&Li´s D, Fu&Li´s F, $p < 0.02$ and Tajima´s D, $p > 0.10$.

Molecular marker	**Ns**	**S**	**Nh**	**Hd**	**π**	**Fu & Li´s D**	**Fu & Li´s F**	**Tajima´s D**
Cytochrome b	450	269	290	0.9760	0.024	**-6.37263**	**-4.18465**	-1.19850

The haplotype network of the cytochrome b has divided the haplotypes into four separated haplotype groups, "A", "B", "C" and "D" (see the Fig. 7). The haplotype group "A" consists of

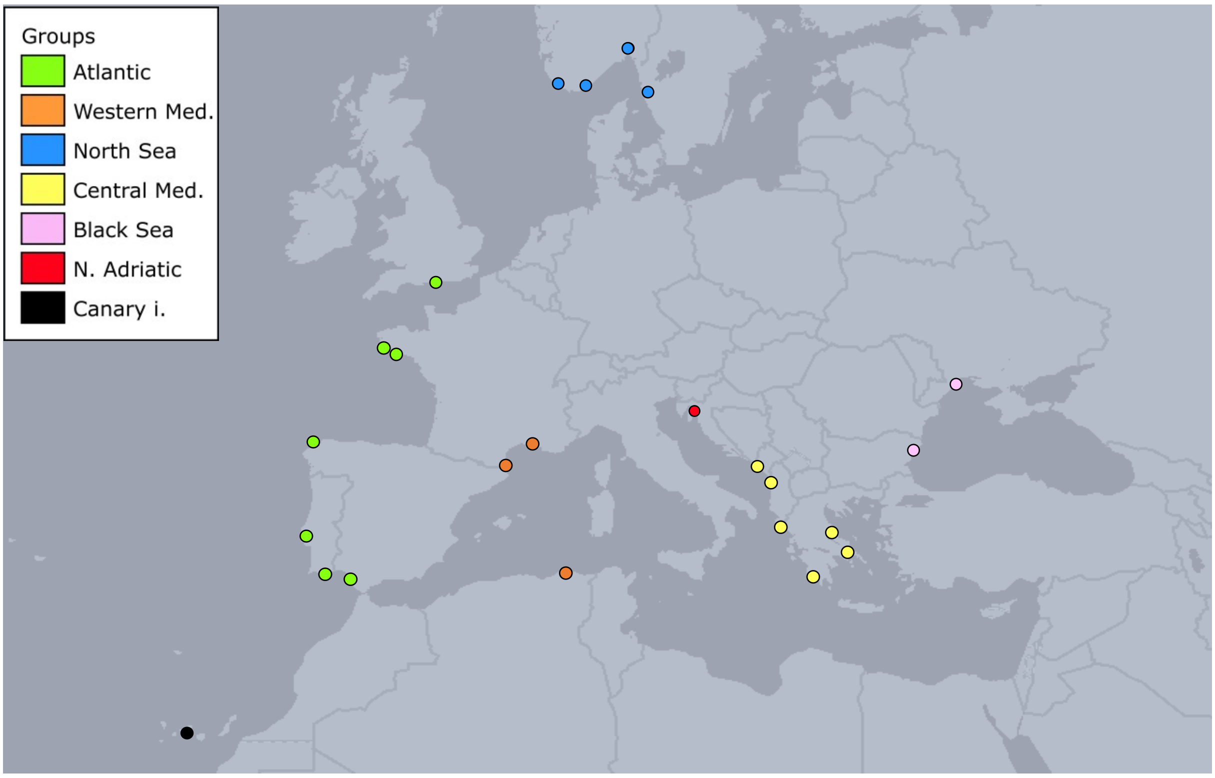

Figure 6: Map showing the division of the localities according to the geographic position. The colours correspond to the regions as described in the legend. Some of the localities were merged due to the proximity of neighbourings.

the haplotypes found in the individuals inhabiting the North Sea, the Atlantic Ocean, and the western Mediterranean. The central part of the haplotype network of the haplotype group "A" is one haplotype shared among 64 individuals from the North Sea and from the Atlantic Ocean, and also it is shared by one individual from the western Mediterranean. The rest of the network has a "star-like" shape and consist of numerous singletons or haplotypes shared among a few individuals, which are connected to the central haplotype. The population from the western Mediterranean is sharing two haplotypes with the individuals from the Atlantic Ocean (including the central haplotype), one haplotype is shared among two individuals from the

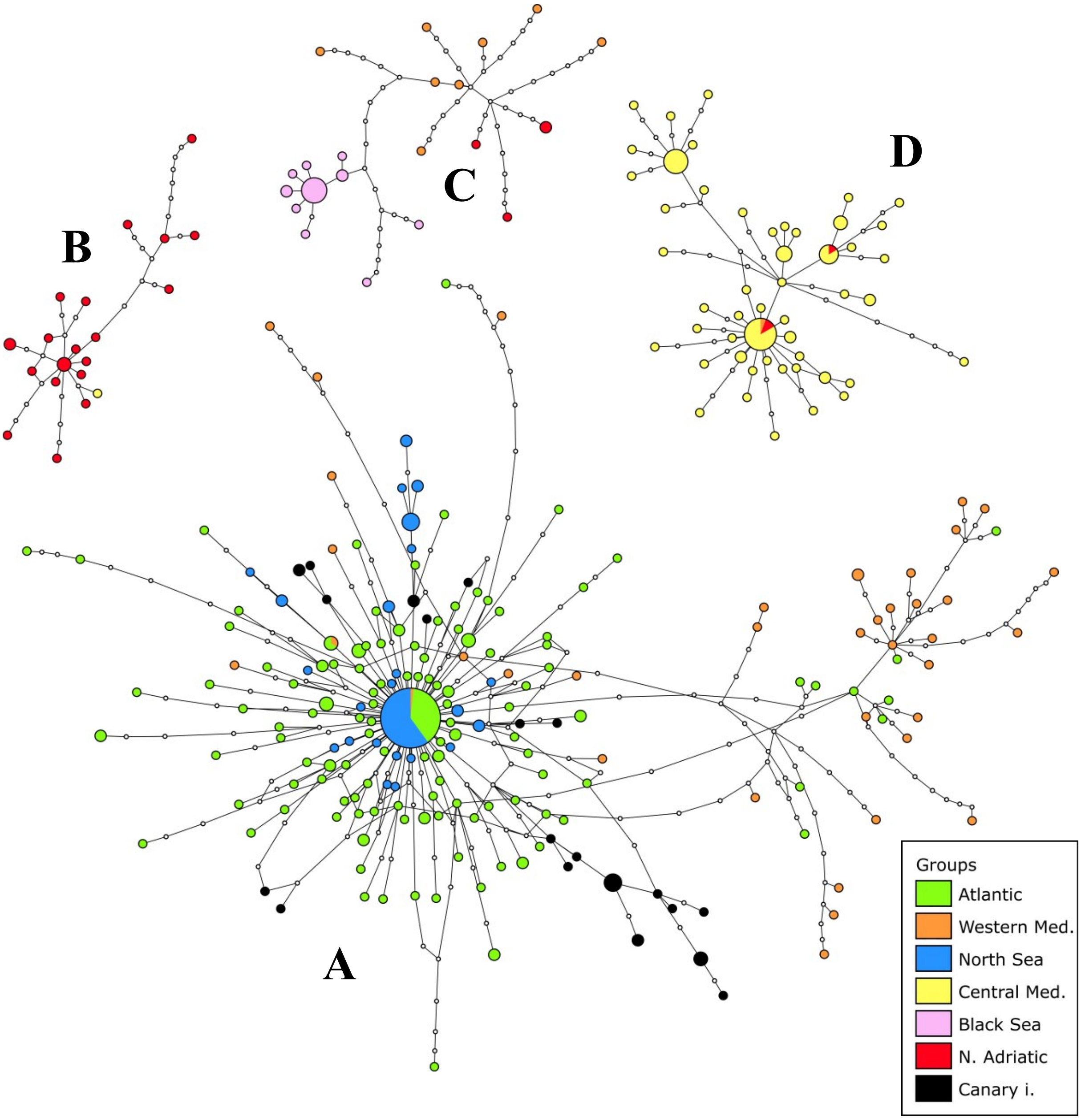

Figure 7: Haplotype network of cytochrome b of the *G. niger*. The colours correspond to the regions as described in legend and in the map (see the Fig. 6), area of the circles reflects the frequency of haplotype. The letters serve to distinguish particular haplotype groups. Mutation steps are symbolized with the white circles.

western Mediterranean, and the rest of the haplotypes from the western Mediterranean falling in the haplotype group "A" are singletons. The population from the Canary Islands does not share any haplotype with the rest of the population and the Canary haplotypes are at least two mutation steps far from the central haplotype. The haplotype group "B" consists of the individuals from the north Adriatic Sea, and also it includes one singleton found in the southern Adriatic Sea. Three of the northern Adriatic haplotypes belong also into the haplotype group "C", together with eight western-Mediterranean haplotypes and with the haplotypes of

Table 10: The diversity measures of the cytochrome b per locality. No – code of locality, N – number of sequences, Nh – number of haplotypes, Hd – haplotype diversity, π – nucleotide diversity

No.	**Locality**	**N_{CytB}**	**Haplotype group/s**	**Nh**	**Hd**	**π**
1	Egersund	20	A	10	0.711	0.00145
2	Kristiansand	17	A	7	0.713	0.00139
3	Oslofjord	26	A	14	0.760	0.00131
4	Gullmarn	9	A	4	0.583	0.00136
5	Pilsey Island	27	A	20	0.937	0.00292
6&7	Crozon& Concarneau	31	A	27	0.991	0.00011
8	Galicia	22	A	19	0.974	0.00238
9	Arrábida	27	A	23	0.980	0.00380
10	Troia peninsula	11	A	10	0.982	0.00440
11	Ria formosa	36	A	34	0.995	0.00538
12	Cádiz	9	A	7	0.917	0.00468
13	Tenerife	29	A	20	0.961	0.00413
14	Banyuls	18	A/C/D	18	1.000	0.01847
15	Berre lagoon	7	A	7	1.000	0.00902
16	Annaba	23	A/C	22	0.996	0.01208
17	Soline, Krk	29	B/C/D	24	0.985	0.01610
18&19	Strp&Kostanjica	32	B/D	23	0.950	0.00643
20	Vilunit Velipoje	3	D	3	1.000	0.00234
21&22	Syvota& Moraitika, Korfu Isl.	27	D	16	0.909	0.00338
23	Petalidi	6	D	5	0.933	0.00287
24&25 &26	Paralia Rachon&Kastari&Petali Isl.	19	D	14	0.953	0.00373
27	Odessa	5	C	4	0.900	0.00316
28	Varnensko Lake	17	C	7	0.721	0.00177

the Black Sea origin. Even though they are from the same haplotype group, there are not any shared haplotypes among the Black Sea, northern Adriatic Sea nor the western Mediterranean. The last haplotype group "D" consists of the haplotypes found in the Aegean, Ionian and southern Adriatic Sea (summed up as a "Central Mediterranean") and also, two haplotypes from this haplotype group were found to be shared within three individuals from the northern Adriatic Sea and one of these haplotypes was found also in specimen from the Western Mediterranean Sea. The phylogenetic relation constructed as a maximum likelihood tree revealed the supported monophyly of the lineage containing the haplotype groups "A", "B" and "C", but the relationships within the lineage remained unresolved (Supplement VII). The frequencies of haplotypes belonging to the individual haplotype groups at each geographic unit is depicted on the Fig. 8. Apparently, the haplotypes from more than one different haplotype groups can be found only in two regions, in the western Mediterranean Sea and within the Adriatic Sea. The

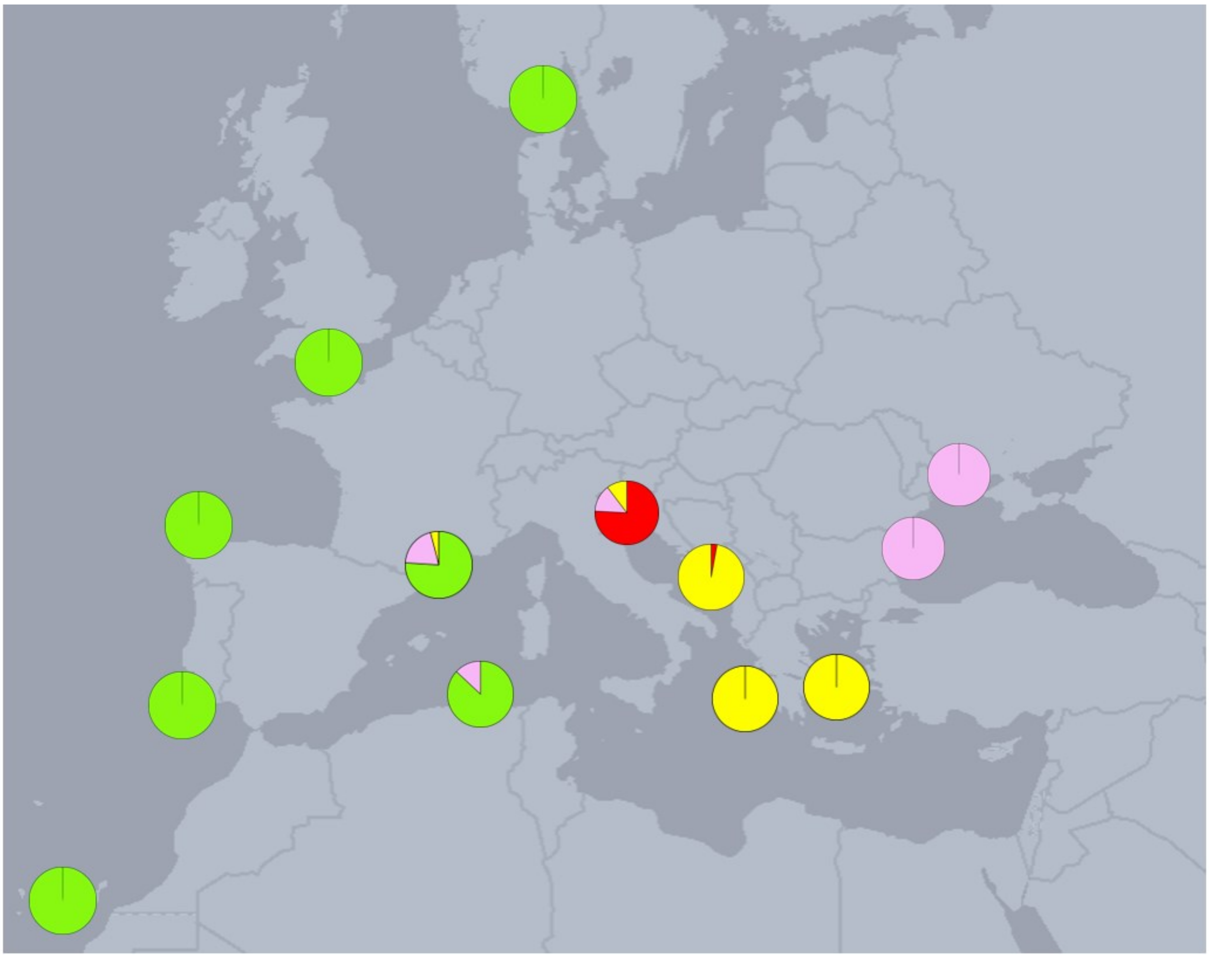

Figure 8: Map showing the frequency of haplotypes from different haplotype groups found on each of the studied regions The colours correspond to the haplotype groups as follows: green – the haplotype group "A", red – the haplotype group "B", pink – the haplotype group "C", yellow – the haplotype group "D"

presence of more than one haplotype group is affecting the molecular diversity of the locality, as it increases its´ nucleotide diversity (see the Tab. 10).

The uncorrected p-distances were computed to evaluate the average molecular differences between haplotype groups and also to see the variability within them. The biggest mean difference (5.5 %) was found between the “A” and “D” haplotype groups, and very high mean difference was also detected between the “B” and “D” haplotype groups (5.2 %). On the other hand, the lowest mean difference was found to be between the “B” and “C” haplotype groups (2.0 %). The highest mean difference within the haplogroups was detected for the “C” haplotype group (0.7 %), for the rest of the haplotype groups the mean p-distance found was below 0.5 %, see the results in the Tab. 11. The mean global molecular difference is 2.4 %, with the highest pairwise distance (6.1 %) found to be between two pairs of sequences, from Canary Islands and Vilunit Velipoje (no. 13. And 20) and from Arrábida and Petalidi (no. 9 and 23) (see the Supplement I and II)

Table 11: average p-distances between and within haplotype groups of cytochrome b. The mean values of p-distances between and within haplotype groups are in bold on left, with maximum and minimum p-distances found between individual localities on right. The codes of the haplotype groups correspond to the figure 7. The values are given in %.

p-distances	A	B	C	D	Within groups
A	*				1.579 **0.440** 0.000
B	3.509 **2.779** 2.105	*			1.228 **0.437** 0.000
C	3.509 **2.737** 1.930	2.456 **1.955** 1.404	*		1.404 **0.690** 0.000
D	6.140 **5.479** 4.912	5.702 **5.173** 4.649	5.351 **4.949** 4.386	*	0.877 **0.348** 0.000

When comparing the regions based on haplotype network result (Atlantic Ocean & western Mediterranean, north Adriatic Sea, the Black Sea and the central Mediterranean), where in the case of more than one haplogroup appearing at the same locality the locality was assigned to the region where most of its sequences fall, most of the observed genetic variability of the cytochrome b can be explained as a variance among populations (86.06 %). The population fixation index F_{ST} went significant ($P<0.01$) and it shows striking structure of the population ($F_{ST} = 0.879$). (see the Tab. 12).

The group pairwise F_{STs} between the regions (Atlantic Ocean & western Mediterranean, north Adriatic Sea, the Black Sea and the central Mediterranean) show strikingly pronounced differentiation of the population. The highest values were observed between the central Mediterranean and the others, but even the rest of the comparations finished with very high values (see the Tab. 13). The lowest F_{ST} value (0.566) was found between the north Adriatic and the Black seas.

Table 12: AMOVA results for the regions Atlantic Ocean & western Mediterranean, north Adriatic Sea, the Black Sea and the central Mediterranean; F_{ST} $P<0.05$

AMOVA	Degrees of freedom	Variance comp.	% of variation	F_{ST}
Among groups	3	21.8109	86.06	**0.879**
Among populations within groups	19	0.47863	1.89	
within populations	427	3.05407	12.05	

Table 13: Pairwise F_{STs} among regions Atlantic Ocean & western Mediterranean, north Adriatic Sea, the Black Sea and the central Mediterranean. The values significant after Bonferroni correction are shown in bold.

Pair. F_{STs} among groups	Atlantic & western Mediterranean	northern Adriatic	Black Sea	Central Mediterranean
Atlantic & western Mediterranean	0.000			
northern Adriatic	**0.743**	0.000		
Black Sea	**0.816**	**0.566**	0.000	
Central Mediterranean	**0.898**	**0.825**	**0.917**	0.000

The second AMOVA was calculated among the geographic regions based on the haplotype network result, but with the deeper subdivision of the “Atlantic Ocean” region into five geographic subregions (see the Tab. 8). The groups compared are: The North Sea, the English Channel & Bay of Biscay, the Iberian basin, the Canary Islands, the western Mediterranean, the north Adriatic Sea, the Black Sea and the central Mediterranean. The majority of variance composition explained by the variance among groups (80.44%), which is less than in the case of the first AMOVA, where the localities from the Atlantic Ocean and the Western Mediterranean were included in one single geographic group. On the other hand, this geographically-based division has increased the importance of the variance within populations, from 12.05 % in the first AMOVA to 19.24 % in the second AMOVA. The fixation index F_{ST} went significant ($P<0.01$) and it shows pronounced structure of the population ($F_{ST} = 0.808$) (see the Tab. 14)

The pairwise F_{STs} between regions (Tab. 15) show significant high values, with exception of the populations from the North Sea, English Channel & Bay of Biscay and Iberian basin, which did not show genetic differentiation (see the Tab. 15). The population from the Canary Islands is moderately differentiated from the rest of the regions of the Atlantic Ocean, with the lowest difference to be between the Canary Islands and the Iberian basin (0.132). Apparently, the lower difference found between the Black Sea and the western Mediterranean (0.614) has masked the high differentiation of the Black Sea population from some of the Atlantic regions (see the Tab. 13 and 15 and Supplement V for comparison). When the Atlantic region is divided, the highest differentiation (0.947) is between the Black Sea and the North Sea, but also the central Mediterranean remain highly differentiated from all the other regions.

Table 14: AMOVA results for the regions North Sea, English Channel & Bay of Biscay, Iberian basin, Canary Islands, western Mediterranean, north Adriatic Sea, the Black Sea and the central Mediterranean; F_{ST} $P<0.05$

AMOVA	Degrees of freedom	Variance comp.	% of variation	F_{ST}
Among groups	7	12.767	80.44	**0.808**
Among populations within groups	15	0.050	0.31	
within populations	427	3.054	19.24	

Table 15: Pairwise F_{STs}, among the regions North Sea, English Channel & Bay of Biscay, Iberian basin, Canary Islands, western Mediterranean, north Adriatic Sea, the Black Sea and the central Mediterranean; The values significant after Bonferroni correction are shown in bold.

Pairwise F_{STs}	North Sea	E. Channel &B. Biscay	Iberia	Canary I.	W. Medit.	northern Adriatic	Black Sea	Central Med.
North Sea	0.000							
E. Channel &B. of Biscay	**0.025**	0.000						
Iberia	**0.026**	0.003	0.000					
Canary I.	**0.247**	**0.156**	**0.132**	0.000				
W. Medit.	**0.339**	**0.267**	**0.265**	**0.255**	0.000			
northern Adriatic	**0.796**	**0.741**	**0.751**	**0.677**	**0.452**	0.000		
Black Sea	**0.947**	**0.908**	**0.871**	**0.892**	**0.614**	**0.566**	0.000	
Central Mediterranean	**0.941**	**0.927**	**0.918**	**0.918**	**0.837**	**0.825**	**0.917**	0.000

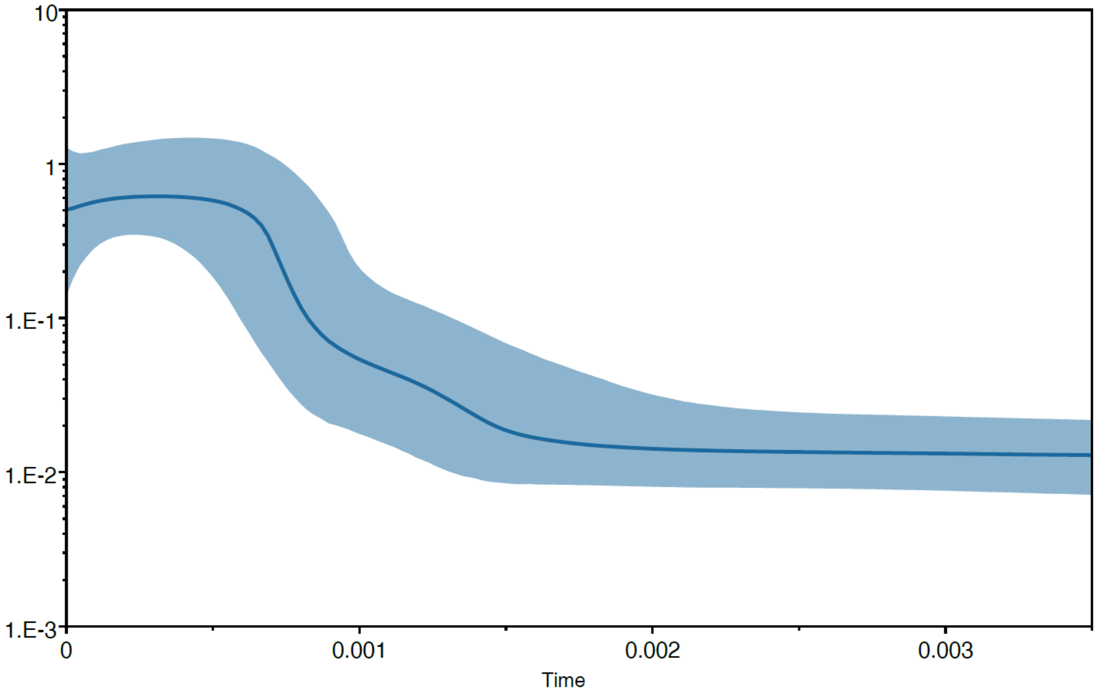

Figure 9: Bayesian skyline plot for *G. niger* for cytochrome b. The curve describes the fluctuation of effective population size from recent to the coalescence from left to right. The line represents median estimate and the blue area shows the 95% confidence interval. The Y-axis shows the effective population size under logarithmic transformation. The X-axis shows the time in units of mutation per nucleotide position.

The Mantel test of isolation by distance (Supplement IX) comparing the pairwise F_{STs} between localities with the shortest sea distances among them finished with significantly positive result, indicating existing, although low correlation ($R^2 = 0.3812$) between geographic distance and the molecular distance between localities. Anyway, the distribution of values is does not have linear course, as the values of F_{STs} are rather close to zero among the closer localities, whereas with the distance over 3000 kilometres, the molecular distance appear to be over 0.8.

The Bayesian Skyline plot shows rapid increase of the effective population size in the past after a long stagnation, followed by the stagnation and a moderate recent decrease (see the Fig. 9).

In order to have a closer look in detail into each region and to reveal whether there is a finer-scale structure within each region, the separate analysis of each geographic region was performed separately.

4.1.1: Atlantic Ocean and the North Sea

A total of 264 individuals from the nine Atlantic and four North Sea localities was analysed. The high haplotype diversity (Hd = 0.9404) together with very low nucleotide diversity (π = 0.00327) correspond to highly statistically significant negative values of neutrality tests (Tajima´s D $p < 0.0001$) suggesting a recent population expansion or selective sweep (see the Tab. 16).

Table 16: Diversity measures of cytochrome b of the Atlantic and the North Sea populations of *G. niger*. Ns – number of sequences, S – number of segregating sites, Nh – number of haplotypes, Hd – haplotype diversity, π – nucleotide diversity, Fu&Li´s D, Fu&Li´s F and Tajima´s D. Statistical significance of Fu&Li´s D, Fu&Li´s F, $p < 0.02$ and Tajima´s D, $p < 0.0001$.

Population	**Ns**	**S**	**Nh**	**Hd**	**π**	**Fu & Li´s D**	**Fu & Li´s F**	**Tajima´s D**
Atlantic & North Sea	264	171	163	0.9404	0.00327	**-7.14114**	**-5.86644**	**-2.71563**

The haplotypes found in the samples from the Atlantic Ocean and the North Sea do belong into the haplotype group A, without any exception. The star-shape of the haplotype network (Fig. 10) is in accordance with negative value of Tajima´s D, probably as a result of bottleneck effect caused by the climatic oscillations (see discussion). The central haplotype shared among 64 individuals can often be found in the individuals from the North Sea, and also it is present in the samples from the English Channel, the Bay of Biscay and also from all of the Iberian localities. Rather than the one common shared haplotype, in the Iberian, Biscay and English localities prevail the haplotypes which are derived from the central one, and these haplotypes remain poorly inter-connected among each other rather than through the central haplotype. Only a few haplotypes are shared among localities, and only four haplotypes (including the central one) are shared among geographic subregions. The Iberian haplotypes seem to be more differentiated, with frequent multiple mutation step distance from the central haplotype and between each other.

No haplotypes from the Canary Islands are shared with other localities, and their position in the haplotype network may indicate separation with repeated contact with the rest of the Atlantic population. Interestingly, there is one haplotype with multiple occurrence found in the three localities from the North Sea, which is separated from the central haplotype by three mutation steps. Moreover, this haplotype is closely connected to three other haplotypes, which are even more far from the central haplotype of the network.

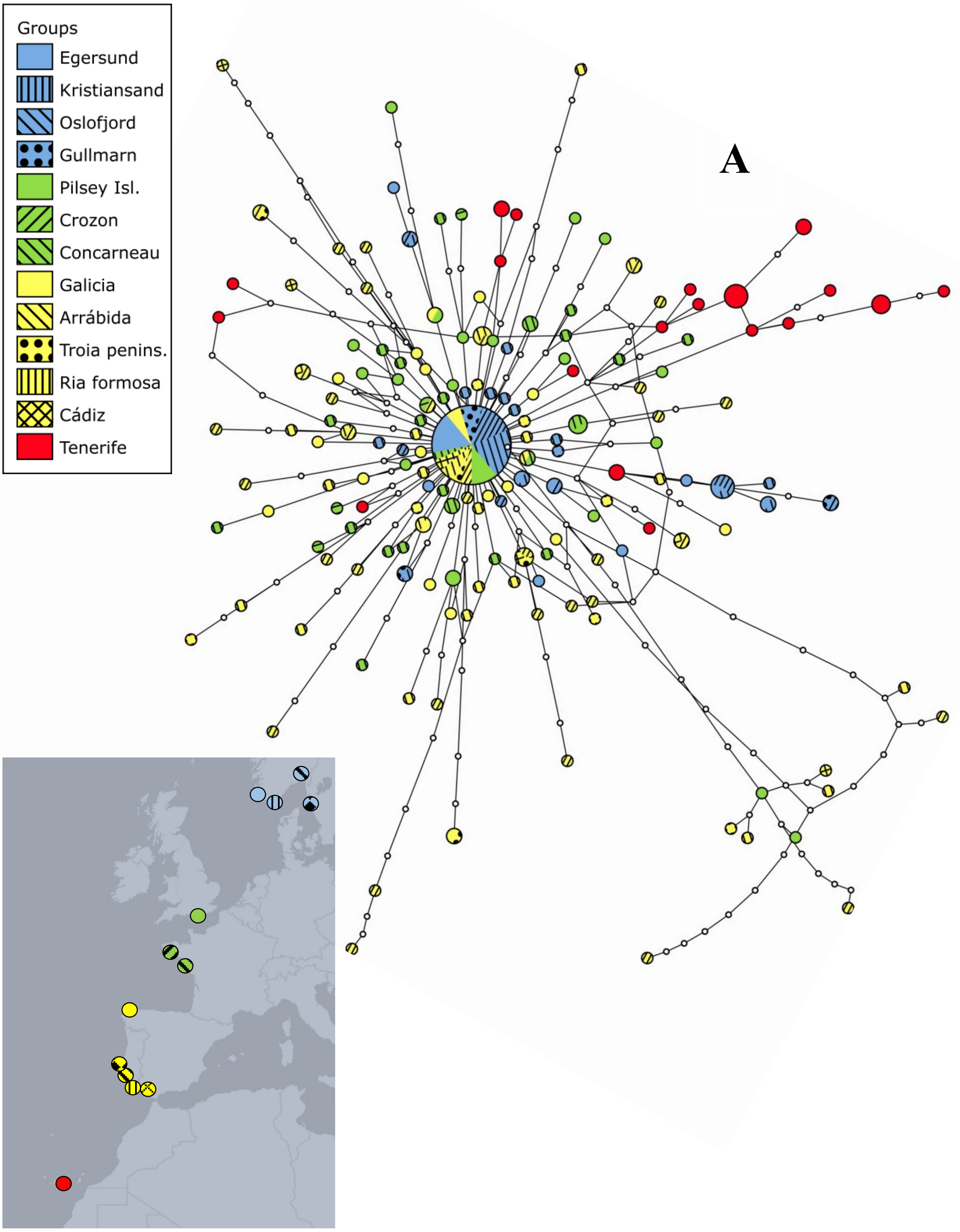

Figure 10: Haplotype network of cytochrome b of the Atlantic and the North Sea populations of *G. niger*. The colours correspond to the regions as follows: blue – the North Sea, green – English Channel & Bay of Biscay, yellow – Iberian basin, red – the Canary Islands, area of the circles reflects the haplotype frequency. The letter serves to distinguish particular haplotype group, as in the Fig. 7. Mutation steps are symbolized with the white circles.

Table 17: Average uncorrected p-distances between and within populations from the regions of Atlantic Ocean and the North Sea. The mean values of p-distances between and within regions are in bold, with maximum and minimum p-distances found between each pair of particular localities. The values are given in %.

p-distances	North Sea	E. Channel & B. Biscay	Iberia	Canary Islands	Within groups
North Sea	*				0.614 **0.136** 0.000
E. Ch. & B. Biscay	1.053 **0.210** 0.000	*			1.140 **0.275** 0.000
Iberia	1.404 **0.310** 0.000	1.491 **0.373** 0.000	*		1.842 **0.463** 0.000
Canary Islands	0.700 **0.341** 0.088	1.228 **0.405** 0.088	1.579 **0.503** 0.088	*	0.877 **0.413** 0.000

The computation of uncorrected p-distances (see the Tab. 17) between and within regions revealed that the differences between regions are increasing in the southward direction, which is in accordance with the analysis discussed above. Interestingly, the highest distance found between all pairs of sequences was 1.8 %, and this difference was found between two samples from the locality no. 11 (see the Supplement II), which belongs in the Iberian group. The within region distances also follow the trend of increasing diversity with the southward direction, although the Iberian population shows higher mean intrapopulation differences (0.46 %) than the population from Canary Islands (0.41 %).

Table 18: AMOVA results for the regions North Sea, English Channel & Bay of Biscay, Iberian basin, Canary Islands; F_{ST} $P<0.05$

AMOVA	Degrees of freedom	Variance components	% of variation	F_{ST}
among groups	3	0.127	6.70	**0.061**
Among populations within groups	8	-0.011	-0.58	
within populations	252	1.785	93.88	

Table 19: Pairwise F_{STs} among the geographic regions: North Sea, English Channel & Bay of Biscay, Iberian basin, Canary Islands. The values significant after Bonferroni correction are shown in bold.

Pair. F_{STs} among groups	North Sea	E. Channel & B. Biscay	Iberia	Canary Islands
North Sea	0.000			
E. Channel & B. Biscay	**0.025**	0.000		
Iberia	**0.026**	0.003	0.000	
Canary Islands	**0.247**	**0.156**	**0.132**	0.000

The majority (93.88 %) of the variance of the Atlantic and the North Sea population can be explained by the variation within particular localities. Only 6.7 % of the variation can be explained by the differences between groups (North Sea, English Channel & Bay of Biscay, Iberian basin and Canary Islands) (See the Tab. 18). This finding is supported by the result of pairwise F_{STs} computation (see the Tab. 19), as the population from the Canary Islands present moderate differentiation from the rest of the population. Also, the North Sea, although sharing the central haplotype with the southern localities, presents weak differentiation from the rest of the population. When the particular localities were compared among each other, only the locality from the Canary Islands show significant difference from the rest (see Supplement V).

The population from the Atlantic Ocean seem to be highly inter-connected with the population from the western Mediterranean (see the Fig. 7). To evaluate the degree of differentiation between the western Mediterranean basin and the Atlantic Ocean, separate AMOVA and F_{STs} computation was performed (see the Tab. 20 and 21).

Table 20: AMOVA results for the regions North Sea, English Channel & Bay of Biscay, Iberian basin, Canary Islands and the western Mediterranean; F_{ST} $P<0.05$

AMOVA	Degrees of freedom	Variance components	% of variation	F_{ST}
among groups	4	0.643	18.73	**0.081**
Among populations within groups	10	0.058	1.70	
within populations	297	2.731	79.57	

Adding of the western Mediterranean population to the model has increased the percentage of variability explained by the group sorting, from the 6.7 % which was counted for the Atlantic population only (see the Tab. 18), to 18.7 % in the case of Atlantic with western Mediterranean (Tab. 20), which resulted from the limited connectivity among the both water bodies. This limited connectivity is apparent also from the pairwise F_{STs}, which revealed that highest differentiation can be found between the western Mediterranean and the North Sea, but the rest of the population (the English Channel & Bay of Biscay, Iberian basin and Canary Islands) have all similar level of differentiation, approximately 0.26 (Tab. 21).

Table 21: Pairwise F_{STs} among the geographic regions: North Sea, English Channel & Bay of Biscay, Iberian basin, Canary Islands and the western Mediterranean. The values significant after Bonferroni correction are shown in bold.

Pairwise F_{STs} among groups	North Sea	E. Channel & B. Biscay	Iberian basin	Canary Islands	Western Mediterran.
North Sea	0.000				
E. Channel & B. Biscay	**0.025**	0.000			
Iberia	**0.026**	0.003	0.000		
Canary Islands	**0.247**	**0.156**	**0.132**	0.000	
Western Mediterran.	**0.339**	**0.267**	**0.265**	**0.255**	0.000

4.1.2: Western Mediterranean

The western Mediterranean subpopulation presents extremely high haplotype diversity (Hd = 0.999), with 47 haplotypes found in 48 samples. Due to the excess of singletons, the Fu and Li´s neutrality tests finished with negative values. The Tajima´s D is also negative, but not significant (see the Table 22).

Table 22: Diversity measures of cytochrome b of eastern Mediterranean population of *G. niger*. Ns – number of sequences, S – number of segregating sites, Nh – number of haplotypes, Hd – haplotype diversity, π – nucleotide diversity, Fu&Li´s D, Fu&Li´s F and Tajima´s D. Statistical significance of Fu&Li´s D, Fu&Li´s F, $p < 0.02$ and Tajima´s $p > 0.05$.

Population	Ns	S	Nh	Hd	π	Fu & Li´s D	Fu & Li´s F	Tajima´s D
W. Mediterranean	48	134	47	0.999	0.0145	**-3.42894**	**-3.35016**	-1.74940

The haplotype network (Fig. 11) shows extreme differentiation of the western Mediterranean population, with only one haplotype shared between two individuals from Annaba (no. 16, Algeria) and the rest being found as singletons. 38 haplotypes found in all of the three localities in the western Mediterranean belong into the haplotype group "A", together with the haplotypes from the Atlantic Ocean. Three samples from the Annaba (locality no. 16, Algeria) and four samples from Banyuls (no. 14, France) belong in the haplotype group "C" with the haplotypes from the Black Sea, and one sample from Banyuls bear a haplotype from the haplotype group "D" which predominates in the Ionian and southern Adriatic Seas. The individuals from Berre lagoon (no. 15, France) belong into the haplotype group "A" only.

Geographically, the western Mediterranean was divided to the south represented by Annaba (no. 16, Algeria) and north represented by Banyuls and Berre Lagoon (no. 14 and 15, France). This division corresponds with the hydrological patters found in the western Mediterranean subbasin. The mean within region p-distance is 1.2 % in the south and 1.6 % in the north, but the range of distances is higher in the north, which results from the presence of three haplotype groups found in Banyuls (see the Tab. 23). When comparing the particular haplotype groups, the highest within group distance can be found between samples from Annaba and from Banyuls (5.7%, see the Tab. 24 and also the Supplement II). Interestingly, the haplotype group "C" presents also high within group distance, with the highest value found between two samples from Banyuls (1.3 %). The haplotype group "D" is far more differentiated (see the Tab. 24), which agrees with the previous analysis (see the Tab. 11).

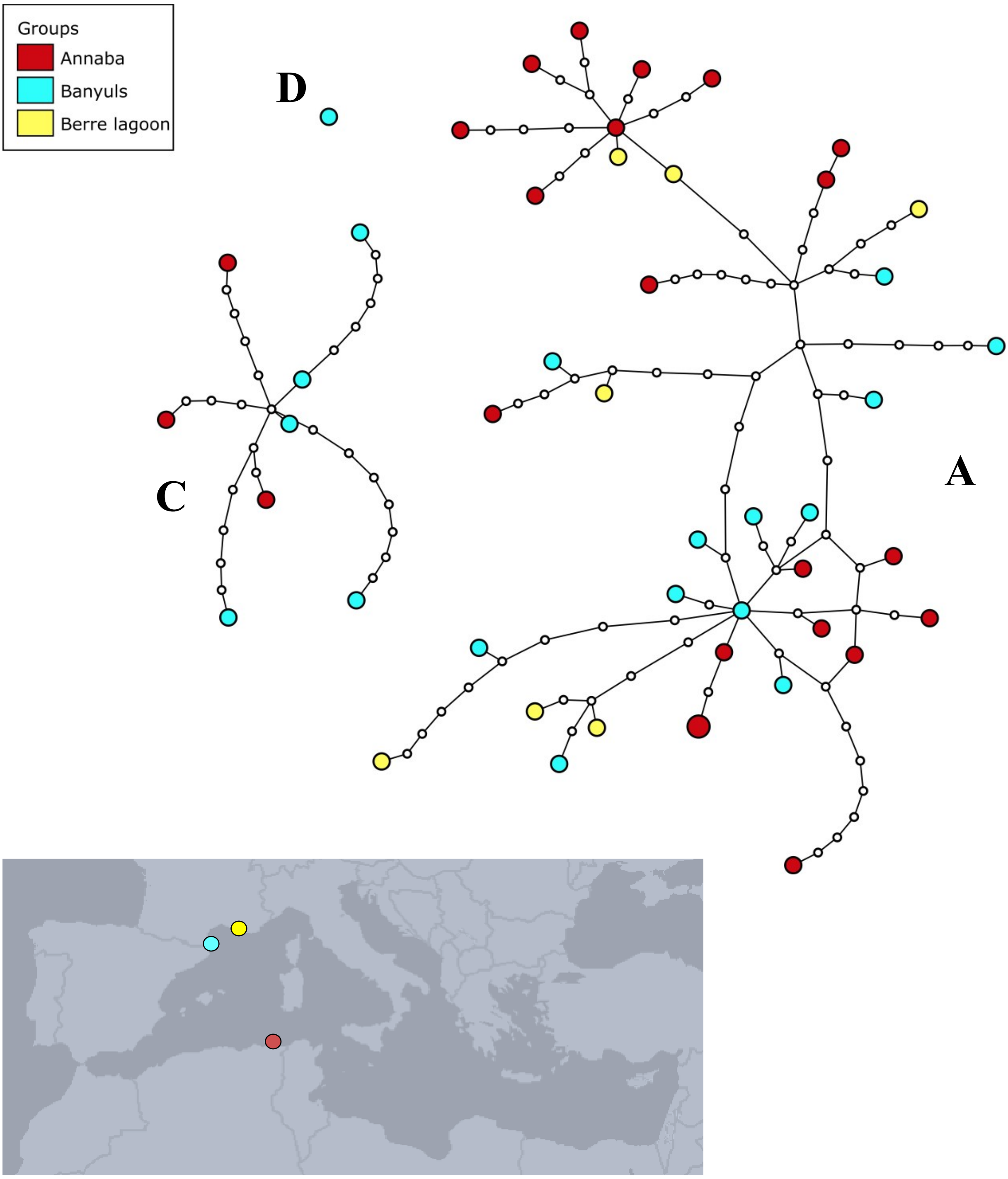

Figure 11: Haplotype network of cytochrome b of western Mediterranean population of *G. niger*. The colours correspond to the regions as described in legend, area of the circles reflects the frequency of haplotype. The letters serve to distinguish particular haplotype groups as in the Fig. 7. Mutation steps are symbolized with the white circles.

Table 23: Average p-distances between and within geographic groups of western Mediterranean population. The locality Annaba (no. 16) represents the south western Mediterranean, whereas the localities Banyuls (no. 14) and Berre lagoon (no. 15) represent the north western Mediterranean. The mean values of p-distances between and within localities are in bold, with maximum and minimum p-distances found between each pair of particular localities. The values are given in %.

p-distances among geographic groups	Annaba	Banyuls & Berre lagoon	Within groups
Annaba	*		2.807 **1.208** 0.000
Banyuls & Berre lagoon	5.702 **1.443** 0.088	*	5.614 **1.647** 0.175

Table 24: Average p-distances between and within haplotype groups found within the western Mediterranean population. The mean values of p-distances between and within haplotype groups are in bold, with maximum and minimum p-distances found between each pair of haplotype groups. The values are given in %.

p-distances	A	C	D	Within groups
A	*			1.491 **0.825** 0.000
C	3.158 **2.401** 1.930	*		1.316 **0.743** 0.175
D	5.702 **5.394** 5.000	5.000 **4.781** 4.649	*	n/a **n/a** n/a

The AMOVA calculated between north and south of the western Mediterranean (see the Tab. 25) has confirmed that neither the geographic origin nor the difference between the north and the south of the basin explains the given molecular variability. Most variability was given by the variability within populations. The global F_{ST} is low and not significant, neither are the pairwise F_{STs} calculated between the two regions (see the Tab. 26), probably as a result of high haplotype diversity in the samples without any detectable geographic pattern.

Table 25: AMOVA results for the north and south regions of the western Mediterranean Sea. F_{ST} P>0.05

AMOVA	Degrees of freedom	Variance components	% of variation	F_{ST}
among groups	1	-0.503	-6.08	0.030
Among populations within groups	1	0.747	9.03	
within populations	45	8.027	97.05	

Table 26.: Pairwise F_{STs} among the northern and southern population of the western Mediterranean Sea. The values significant after Bonferroni correction are shown in bold.

Pair. F_{STs} subregions	Banyuls & Berre lagoon	Annaba
Banyuls & Berre lagoon	0.000	
Annaba	0.010	0.000

4.1.3: Northern Adriatic Sea

From the northern Adriatic Sea, 29 individuals from one locality were obtained (see Tab. 27). The subpopulation shows very high haplotype diversity (Hd = 0.985) and high number of segregating sites (S = 96). The tests of neutrality finished with nonsignificant results.

Table 27: Diversity measures of cytochrome b of northern Adriatic population of *G. niger*. Ns – number of sequences, S – number of segregating sites, Nh – number of haplotypes, Hd – haplotype diversity, π – nucleotide diversity, Fu&Li´s D, Fu&Li´s F and Tajima´s D. Statistical significance of Fu&Li´s D, Fu&Li´s F, $p > 0.10$ and Tajima´s D $p > 0.10$.

Population	**Ns**	**S**	**Nh**	**Hd**	**π**	**Fu & Li´s D**	**Fu & Li´s F**	**Tajima´s D**
N. Adriatic	29	96	24	0.985	0.0161	-0.03100	-0.41008	-0.98685

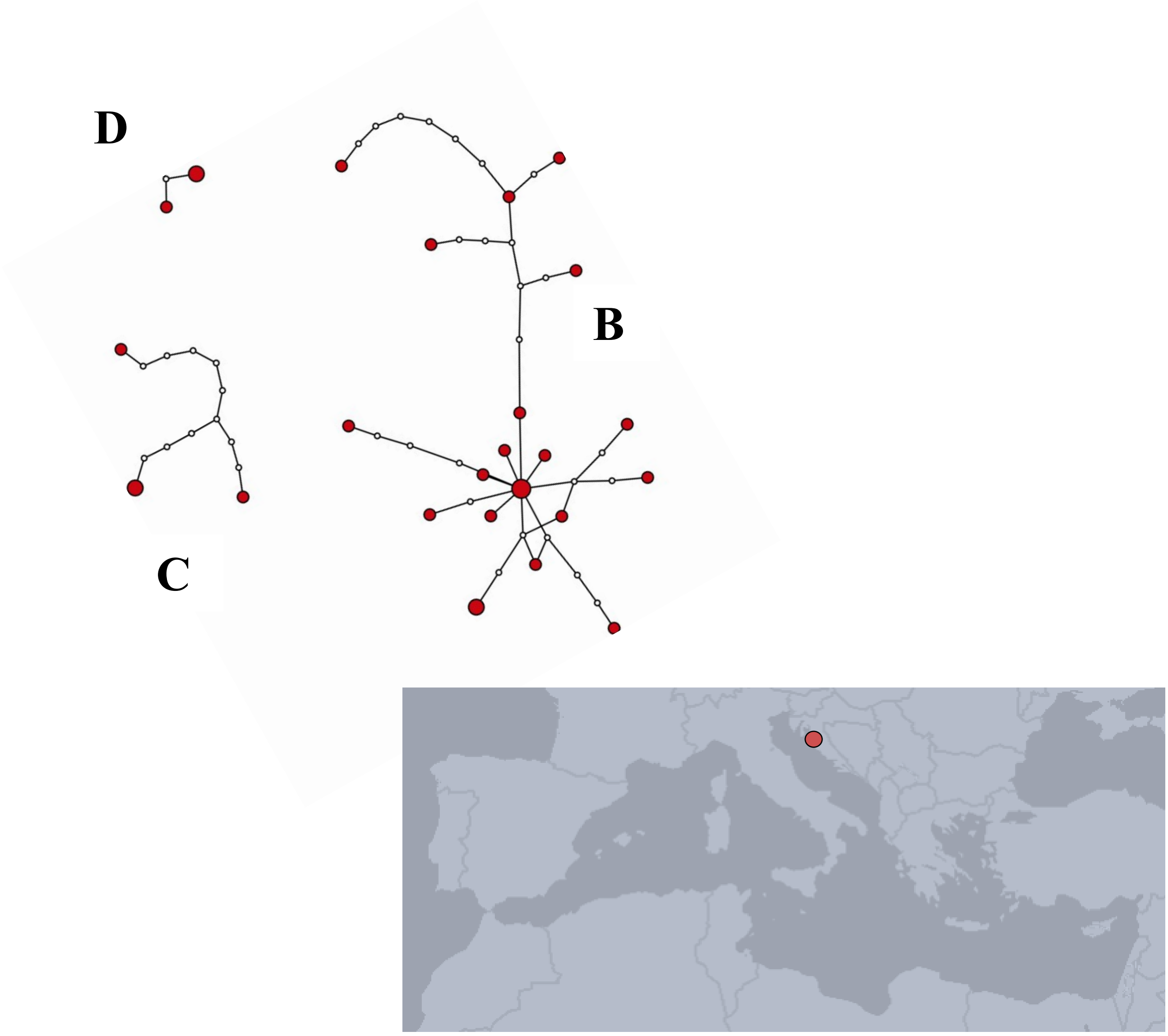

Figure 12: The haplotype network of cytochrome b of the northern Adriatic population of *G. niger*. Area of the circles reflects the haplotype frequency. The letter serves to distinguish particular haplotype group as in the Fig. 7. Mutation steps are symbolized with the white circles. Map is showing the geographic position of the locality in the Adriatic Sea.

Table 28: Diversity measures of cytochrome b of haplotype group “B” part of the northern Adriatic population. Ns – number of sequences, S – number of segregating sites, Nh – number of haplotypes, Hd – haplotype diversity, π – nucleotide diversity, Fu&Li´s D, Fu&Li´s F and Tajima´s D. Statistical significance of Fu&Li´s D, Fu&Li´s F, < 0.05 and Tajima´s D < 0.05.

Population	**Ns**	**S**	**Nh**	**Hd**	**π**	**Fu & Li´s D**	**Fu & Li´s F**	**Tajima´s D**
Haplgroup “B”	23	38	20	0.984	0.0044	**-2.64359**	**-2.87848**	**-2.04462**

To evaluate better the population dynamics within the haplotype group “B”, the separate analysis of diversity and computation of neutrality tests was conducted only for this part of population (Tab. 28). In this case, all the neutrality tests finished with significant negative result, indicating that the population underwent population expansion or selection sweep.

The haplotype network (Fig. 12) has revealed the presence of three distinct haplotype groups. The affiliation of the distinct haplotype groups found among the northern Adriatic samples to the haplotype groups found in the rest of the studied area is delineated by the letters in the haplotype network (see Fig. 7). The majority of the samples belong into the haplotype group “B”, which is a unique haplotype group found only in the Adriatic Sea. The three haplotypes from the haplotype group “C” were found in four individuals, and the two haplotypes from the group “D” only in three individuals from the northern Adriatic locality (Fig. 12, see also the Fig. 8).

The uncorrected p-distances between the three haplotype groups were calculated (see Tab. 29). The biggest difference was found between the haplotype group “B” and “D” (5.1 %), which is in accordance with the distance between haplotype groups calculated from the entire dataset (see the Tab. 11). The average within group distance of the prevailing Adriatic haplotype group “B” was 0.4 %, with 1.2 % as a highest distance found between pairs of sequences. Interestingly, the haplotype group “C” presents high within group distance (0.6 %), which was found only between four samples falling into this haplogroup.

Table 29: Average p-distances between and within groups of the northern Adriatic Sea. The mean values of p-distances between and within haplotype groups are in bold, with maximum and minimum p-distances found between haplotype groups. The values are given in %.

p-distances	B	C	D	Within groups
B	*			**0.440** 1.228 0.000
C	**1.734** 2.105 1.404	*		**0.629** 0.877 0.000
D	**5.125** 5.526 4.649	**4.744** 5.000 4.386	*	**0.117** 0.175 0.000

4.1.4: The Black Sea

A total of 22 individuals from the two localities from the Black Sea were analysed (see the Tab. 30). The individuals show quite high haplotype diversity (Hd = 0.753) but relatively low number of segregating sites (S = 18), compared to the other subpopulations. The individuals from the two localities (Odessa Bay and Varnensko Lake) do not show any division based on the geography and they share the most frequent haplotype (see the Fig. 13). The existence of one shared haplotype with the star-like pattern is in accordance with the significant negative Tajima´s D, indicating recent population expansion or recent selective sweep (see the Tab. 30 for more details), however the nonsignificant Fu & Li´s statistics indicate no deviation from the neutral expectation.

Table 30: Diversity measures of cytochrome b of the Black Sea population of *G. niger*. Ns – number of sequences, S – number of segregating sites, Nh – number of haplotypes, Hd – haplotype diversity, π – nucleotide diversity, Fu&Li´s D, Fu&Li´s F and Tajima´s D. Statistical significance of Fu&Li´s D, Fu&Li´s F, $p > 0.05$ and Tajima´s D, $p < 0.05$.

Population	**Ns**	**S**	**Nh**	**Hd**	**π**	**Fu & Li´s D**	**Fu & Li´s F**	**Tajima´s D**
Black Sea	22	18	10	0.753	0.00204	-2.07015	-2.36961	**-1.94674**

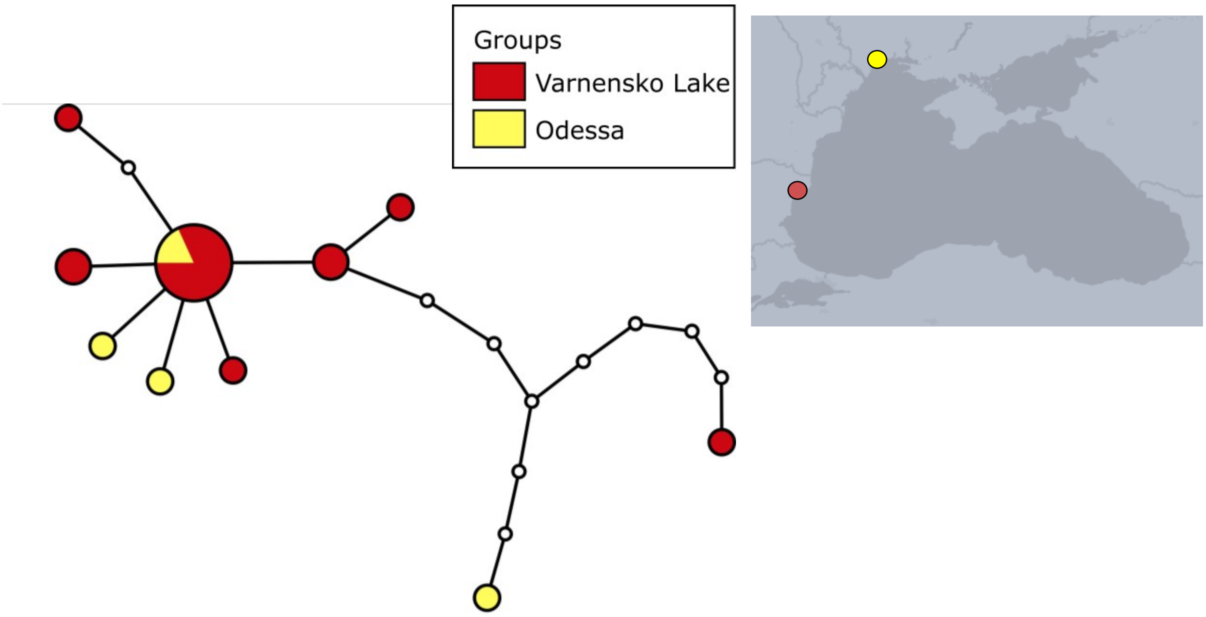

Figure 13: The haplotype network of cytochrome b of the Black Sea population of *G. niger*. The colours correspond to the localities as in the legend and in the map, area of the circles reflects the haplotype frequency. The letter serves to distinguish particular haplotype group. Mutation steps are symbolized with the white circles. Map showing the geographic position of the localities in the Black Sea.

The pairwise p-distance (see the Tab. 31) revealed that the divergence within the Odessa population (0.3 %) is higher than the average difference between populations (0.2 %), and it is in accordance with the nucleotide diversity (0.0018) found in Varnensko Lake, which is lower than the nucleotide diversity in the Odessa Bay (0.0032), see the Tab. 10. Moreover, the highest pairwise p-distance found, is between two samples from Varnensko Lake (1.0 %). This value is higher than the maximum p-distance between the two localities (0.9 %). The reliability of these results has to be taken with caution because of the low number of samples studied from the Black Sea localities.

Table 31: Pairwise p-distances between and within localities of the Black Sea. The mean value of p-distance between and within localities is in bold, with maximum and minimum p-distances found between localities. The values are given in %.

p-distances	Odessa	Varnensko Lake	Within groups
Odessa	*		**0.316** 0.701 0.000
Varnensko Lake	**0.234** 0.877 0.000	*	**0.177** 0.965 0.000

4.1.5: Central Mediterranean

A total of 86 sequences of cytochrome b of *Gobius niger* from “central Mediterranean” (southern Adriatic, Ionian and Aegean seas) subpopulation were analysed. This subpopulation presents very high haplotype diversity (Hd = 0.953), but the levels of nucleotide diversity (π = 0.00359) are low (see the Tab. 32). The negative values of Fu and Li´s and Tajima´s tests are statistically significant, indicating recent population expansion or recent selective sweep. The negative values of the neutrality tests are caused by the excess of rare singletons and haplotypes over expected neutrality model.

Table 32: Diversity measures of cytochrome b of the central Mediterranean population of *G. niger*. Ns – number of sequences, S – number of segregating sites, Nh – number of haplotypes, Hd – haplotype diversity, π – nucleotide diversity, Fu&Li´s D, Fu&Li´s F and Tajima´s D. Statistical significance of Fu&Li´s D, Fu&Li´s F, $p < 0.02$ and Tajima´s D, $p < 0.01$.

Population	**Ns**	**S**		**Hd**	**π**	**Fu & Li´s D**	**Fu & Li´s F**	**Tajima´s D**
C. Medit.	86	69	50	0.953	0.00359	**-5.41351**	**-4.98948**	**-2.32405**

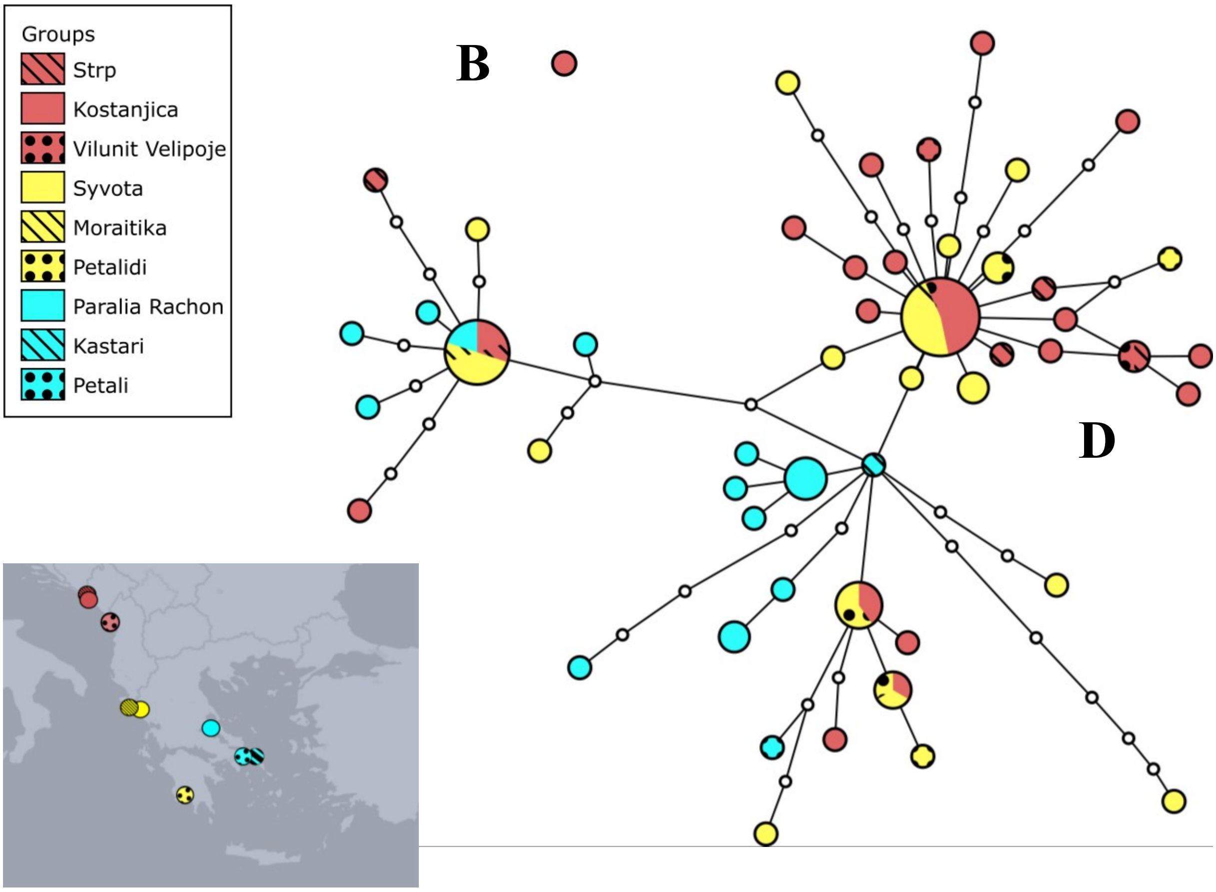

Figure 14: Haplotype network of cytochrome b of “central Mediterranean” population of *G. niger*. The colours correspond to the localities as described in legend and in the map. The colours correspond to the regions as follows: red – southern Adriatic Sea, yellow – Ionian Sea, blue – Aegean Sea, area of the circles reflects the frequency of haplotype. The letters serve to distinguish particular haplotype groups as in the Fig. 7. Mutation steps are symbolized with the white circles.

The haplotype network (Fig. 14) show the complex structure among “central Mediterranean” haplotypes. The Southern Adriatic and Ionian basins share four common haplotypes, and numbers of derived haplotypes (mostly singletons) can be found in both areas. By contrast, only one haplotype is shared among the all three basins, and except this haplotype, there is no other haplotype shared within Aegean Sea area and the rest of the “central Mediterranean”. The star-like shape of the haplotype clusters indicates recent population expansion. One of the samples collected from Kostanjica (locality no. 19, Adriatic Sea) was found to be part of the haplotype group “B” predominated by north Adriatic haplotypes (see the 4.1.3).

The uncorrected p-distances were calculated among the two haplotype groups “B” and “D”, and also among particular geographic groups (southern Adriatic Sea, Ionian Sea and the Aegean Sea). The mean p distance between the haplotype groups “B” and “D” was found to be 5.33%, with the highest difference (5.5 %) laying between the sample from Kostanjica and four samples from the southern Adriatic and Ionian seas (localities no. 19 – 21), which explains the high differentiation found between the southern Adriatic and the Ionian Seas (5,5%) and within the southern Adriatic Sea (mean p-distance 0.6 %, with the maximum 5.5 %), caused by the presence of the sample from the haplotype group “B” in the southern Adriatic Sea. The highest mean p-distance was found between the southern Adriatic and the Aegean seas (0.55 %) (see the Tab. 33).

Table 33: Average p-distances between and within geographic groups of the central Mediterranean region. The mean value of p-distance between and within localities is in bold, with maximum and minimum p-distances found between localities. The values are given in %.

p-distances	Southern Adriatic Sea	Ionian Sea	Aegean Sea	Within groups
Southern Adriatic Sea	*			**0.609** 5.526 0.000
Ionian Sea	**0.478** 5.526 0.000	*		**0.342** 0.877 0.000
Aegean Sea	**0.550** 5.439 0.000	**0.394** 0.877 0.000	*	**0.373** 0.789 0.000

Table 34: AMOVA of the central Mediterranean subpopulation, the composition of populations and groups is described in 4.6.1. F_{ST} P<0.01

AMOVA	Degrees of freedom	Variance components	% of variation	F_{ST}
among groups	2	0.109	3.95	**0.056**
Among populations within groups	2	0.045	1.64	
within populations	82	2.596	94.41	

Table 35: Pairwise F_{STs} of the central-Mediterranean subpopulation. The values significant after Bonferroni correction are shown in bold.

Pairwise F_{STs}	Southern Adriatic Sea	Ionian Sea	Aegean Sea
Southern Adriatic Sea	0.000		
Ionian Sea	0.005	0.000	
Aegean Sea	**0.096**	**0.093**	0.000

The analysis of molecular variance (Tab. 34) has revealed that majority (94.41 %) of the variance is explained by the variability within populations from sampled localities, rather than by the variance among subregions. Anyway, small portion of variation (3.95 %) can be considered as a difference between subregions. The results of the AMOVA correspond to the haplotype network, as there are the five shared haplotypes mentioned above, which makes lower the variance among groups. The global F_{ST} (0.056) indicates that the subpopulation is mildly structured. In the table of pairwise F_{STs} (Tab. 35), the moderate difference of Aegean basin from the rest of the central Mediterranean is corroborated by the significant F_{STs} values.

4.2.: D loop

A total of 535 sequences of D loop part of control region of *Gobius niger* with the total length of 296 base pairs were analysed. The sequences show high haplotype (0.9060) and nucleotide (0.01509) diversity despite the very low number of segregating sites (36). The tests of neutrality of Fu & Li and Tajima´s D finished with nonsignificant result, thus no violation of neutrality model was detected (see the Tab. 36). The diversity measures of individual localities are given in the Tab. 37.

Table 36: Diversity measures of D loop of population of G. Niger. Ns – number of sequences, S – number of segregating sites, Nh – number of haplotypes, Hd – haplotype diversity, π – nucleotide diversity, Fu&Li´s D, Fu&Li´s F and Tajima´s D. Statistical significance of Fu&Li´s D, Fu&Li´s F, p > 0.1 and Tajima´s D, p > 0.1.

Marker	**Ns**	**S**	**Nh**	**Hd**	**π**	**Fu & Li´s D**	**Fu & Li´s F**	**Tajima´s D**
D loop	535	36	147	0.9060	0.01509	-0.99554	-1.08679	-0.78118

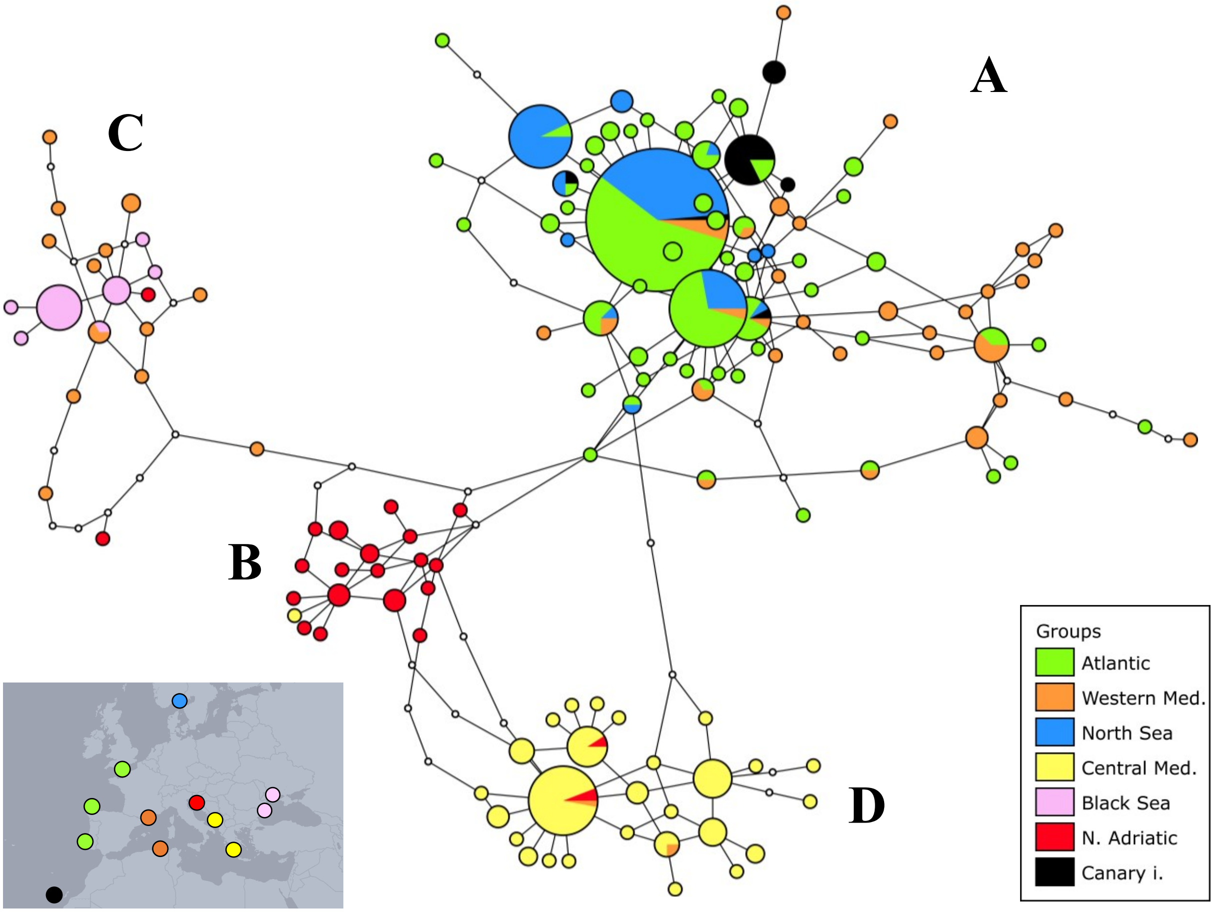

Figure 15: Haplotype network of D loop of the *G. niger*. The colours correspond to the regions as described in legend, approximate location is depicted on the map (exact location is described in the Tab. 3). Area of the circles reflects the frequency of haplotype. The letters serve to distinguish particular haplotype groups as for cytochrome b (Fig. 7). Mutation steps are symbolized with the white circles.

Table 37: The diversity measures of the cytochrome b per locality. No – code of locality, N – number of sequences, Nh – number of haplotypes, Hd – haplotype diversity, π – nucleotide diversity

No.	Locality	N Dloop	Haplotype group/s	Nh	Hd	π
1	Egersund	26	A	4	0.588	0.00226
2	Kristiansand	26	A	4	0.532	0.00266
3	Oslofjord	28	A	6	0.646	0.00284
4	Gullmarn	26	A	7	0.788	0.00424
5	Pilsey Island	28	A	9	0.759	0.00471
6&7	Crozon& Concarneau	31	A	11	0.778	0.00400
8	Galicia	30	A	12	0.841	0.00471
9	Arrábida	33	A	10	0.665	0.00437
10	Troia peninsula	26	A	12	0.840	0.00575
11	Ria formosa	39	A	22	0.887	0.01026
12	Cádiz	11	A	6	0.800	0.00448
13	Tenerife	22	A	6	0.589	0.00281
14	Banyuls	23	A/C/D	19	0.972	0.02227
15	Berre lagoon	7	A	7	1.000	0.01014
16	Annaba	35	A/C	23	0.961	0.01682
17	Soline, Krk	29	B/D	21	0.970	0.01193
18&19	Strp&Kostanjica	32	B/C/D	14	0.722	0.00632
20	Vilunit Velipoje	3	D	1	0.000	0.00000
21&22	Syvota& Moraitika, Korfu Isl.	28	D	13	0.857	0.00724
23	Petalidi	6	D	4	0.800	0.00586
24&25&26	Paralia Rachon&Kastari&Petali Isl.	22	D	11	0.857	0.00661
27	Odessa	4	C	2	0.500	0.00338
28	Varnensko Lake	20	C	6	0.658	0.00327

The haplotype network (see Fig. 15) shows the division of haplotypes into the four haplotype groups. For the analysis of D loop, there was used 85 more specimens than for the analysis of cytochrome b, but he haplotype groups correspond to the haplotype groups of the cytochrome b, thus they were marked with the corresponding letter. In comparison with the cytochrome b haplotype network, the haplotype groups are more tightly interconnected. The central part of the haplotype group “A” consists of one broadly shared haplotype, which is very common in the North Sea and in the North-east Atlantic, but also it appears in two localities in the western Mediterranean and in the population from Canary Islands. The high diversity of singletons from the Atlantic Ocean connected to the central haplotype can be seen. In contrast, only 12 haplotypes occur in the North Sea, from which only four are private. The western Mediterranean population shows extreme diversity of haplotypes which split into two distant haplotype groups, the group “A” and “C” In the haplotype group “A”, the western Mediterranean samples share nine haplotypes with the specimens from the Atlantic Ocean, and the rest of the haplotypes is present as singletons. The haplotype group “C” consists of tweleve haplotypes (mostly singletons) from the western Mediterranean, two singletons from the northern Adriatic Sea and the six private haplotypes shared among the specimens from the Black Sea. Interestingly, in the haplotype group “C”. there is also one haplotype shared between the western Mediterranean and the Black Sea. The haplotype group “B” is endemic to the Adriatic Sea, and it has been found mostly in the northern Adriatic Sea, and it also includes one singleton from the southern Adriatic Sea (locality no. 19, Montenegro). The last haplotype group “D” consists of the haplotypes found in the central Mediterranean Sea, and also, two haplotypes were found to be shared with the three specimens from the northern Adriatic Sea and with one specimen from the western Mediterranean, and another haplotype is shared with western Mediterranean specimen only. The phylogenetic relation constructed as a maximum likelihood tree have not found any statistically supported lineages of haplotypes (Supplement VIII).

Table 38: average p-distances between and within haplotype groups of D loop. The mean values of p-distances between and within haplotype groups are in bold, with maximum and minimum p-distances found between individual localities. The codes of the clusters correspond to the Fig. 15. The values are given in %.

p-distances	A	B	C	D	Within groups
A	*				3.716 **0.668** 0.000
B	3.716 **2.130** 1.351	*			1.689 **0.782** 0.000
C	4.392 **2.708** 1.014	3.716 **2.506** 2.027	*		2.703 **0.870** 0.000
D	4.730 **2.407** 0.000	3.378 **1.951** 1.689	4.054 **2.941** 1.35	*	1.689 **0.660** 0.000

The highest mean difference was found between the “C” and “D” haplotype groups (2.94 %) but the highest p-distance found between all sequences (4.7 %) was between multiple localities from the Atlantic Ocean, western Mediterranean and the "central Mediterranean" regions (see the Supplement III and IV). The biggest within group distance was computed for the haplotype group "C", which is in accordance with the results of p-distances of the cytochrome b. Oppositely, the groups "C" and "D" are not the most distant in the case of Cytochrome b, where the highest average p-distance lays between the haplotype group "A" and "D" (see the Tab. 38).

The AMOVA analysis revealed that the division of population into the four geographically defined regions based on the distribution in the haplotype network (Fig. 15) (Atlantic Ocean & western Mediterranean, north Adriatic Sea, the Black Sea and the central Mediterranean) can explain 65.55 % of variation of the d loop sequences (see the Tab. 39). Also, a big part, the 28.67 % of the variation can be explained by the variance within particular localities ("populations"). The global F_{ST} indicate deep structuring of the population, which corresponds with the haplotype network and with the diversity measures.

Table 39: AMOVA results for the regions Atlantic Ocean & western Mediterranean, north Adriatic Sea, the Black Sea and the central Mediterranean; F_{ST} $P<0.01$

AMOVA	Degrees of freedom	Variance comp.	% of variation	F_{ST}
among groups	3	2.343	65.55	**0.713**
among populations within groups	19	0.207	5.78	
within populations	512	1.025	28.67	

Table 40: Pairwise F_{STs} among regions Atlantic Ocean & western Mediterranean, north Adriatic Sea, the Black Sea and the central Mediterranean. The values significant after Bonferroni correction are shown in bold.

Pair. F_{STs} among groups	Atlantic & western Mediterranean	northern Adriatic	Black Sea	Central Mediterranean
Atlantic & western Mediterranean	0.000			
northern Adriatic	**0.584**	0.000		
Black Sea	**0.717**	**0.681**	0.000	
Central Mediterranean	**0.672**	**0.526**	**0.797**	0.000

The pairwise F_{STs} between the regions (Atlantic Ocean & western Mediterranean, north Adriatic Sea, the Black Sea and the central Mediterranean) show highly pronounced structuring of the population. The highest value was observed between the "central Mediterranean" and the Black Sea regions (0.797), whereas the lowest value was found between the Atlantic & western Mediterranean and the northern Adriatic Sea populations (0.584), see the Tab. 40.

In the second AMOVA, the population was divided according to the haplotype network result into the geographic units, but with the deeper subdivision of the "Atlantic Ocean" region into five geographic groups (see the Tab. 8). The regions compared are: The North Sea, the English Channel & Bay of Biscay, the Iberian basin, the Canary Islands, the western Mediterranean, the north Adriatic Sea, the Black Sea and the central Mediterranean. The majority of the molecular variance can be still explained by the variance among geographic regions (groups) (56.75 %), but the percentage of variation explained by the variance within particular localities (populations) has increased in comparison with the first AMOVA, from the 28.67% to the actual 41.44 % (see the Tab. 39 and 41). The global F_{ST} is significant and it indicate deep structuring of the population.

Table 41: AMOVA results for the regions North Sea, English Channel & Bay of Biscay, Iberian basin, Canary Islands, western Mediterranean, north Adriatic Sea, the Black Sea and the central Mediterranean; F_{ST} P<0.05

AMOVA	Degrees of freedom	Variance comp.	% of variation	F_{ST}
among groups	7	1.403	56.75	**0.713**
among populations within groups	15	0.045	1.80	
within populations	512	1.025	41.44	

Table 42: Pairwise F_{STs}, *among* the regions North Sea, English Channel & Bay of Biscay, Iberian basin, Canary Islands, western Mediterranean, north Adriatic Sea, the Black Sea and the central Mediterranean; The values significant after Bonferroni correction are shown in bold.

Pairwise F_{STs}	North Sea	E. Channel &B. Biscay	Iberian basin	Canary I.	W. Medit.	northern Adriatic	Black Sea	Central Med.
North Sea	0.000							
E. Channel &B. of Biscay	**0.061**	0.000						
Iberian basin	**0.090**	0.022	0.000					
Canary I.	**0.476**	**0.357**	**0.249**	0.000				
W. Medit.	**0.338**	**0.218**	**0.212**	**0.229**	0.000			
northern Adriatic	**0.764**	**0.667**	**0.623**	**0.647**	**0.280**	0.000		
Black Sea	**0.890**	**0.845**	**0.780**	**0.895**	**0.474**	**0.681**	0.000	
Central Mediterranean	**0.794**	**0.746**	**0.708**	**0.771**	**0.553**	**0.526**	**0.797**	0.000

The pairwise F_{STs} between regions (Tab. 42) show significant high values, with exception of the values between the populations from the North Sea, the English Channel & Bay of Biscay and Iberian basin, which were only moderate (see the Tab. 42, and also the Supplement VI for reference). The population from the Canary Islands is highly differentiated from the rest of the regions of the Atlantic Ocean, with the lowest difference to be between the Canary Islands and Iberian basin, which is still high (0.25). Interestingly, the western Mediterranean show lower pairwise F_{STs} with the Noth Sea, English Channel & Bay of Biscay and Iberian basin than the sequences from Canary Islands. The highest F_{STs} are between the Black Sea and the Atlantic regions (Noth Sea, English Channel & Bay of Biscay and Canary Islands), and it is considerably higher than the F_{ST} between the Black Sea and the Central Mediterranean, which presented the highest F_{ST} in the previous comparison. The high pairwise F_{STs} of the Atlantic localities with the Adriatic Sea, central Mediterranean and the Black Sea regions, was probably decreased and masked by the connection to the western Mediterranean, which has the F_{ST} with the Black Sea much lower due to the presence of the same haplotype group (0.47).

The Mantel test of isolation by distance (Supplement X) comparing the pairwise F_{STs} between localities with the shortest sea distances among them finished with significantly positive result, indicating existing correlation ($R^2 = 0.4477$) between geographic distance and the molecular distance between localities.

5: Discussion

The population of the black goby was broadly sampled across the area covering most of the species distribution range (see the Tab. 3 and Fig. 5), and the genetic structure was investigated using two mitochondrial markers, the D loop and the cytochrome b. Despite the commonness of this species in the European coastal waters, this work is the first one focusing on the genetic population structure and phylogeography of the black goby, except the small-scale study of Giovannotti et al. (2009).

5.1: Genetic variability

The molecular analysis of the population of the black goby revealed high within species genetic variability. The highest genetic distance found between pairs of samples is 6.1 % (Tab. 11) for cytochrome b, which is between one sample from Canary Islands and three samples from the southern Adriatic, Ionian and Aegean seas. On the D loop marker, the highest difference found was 4.7 % between samples from Ria Formosa (Portugal) and Syvota (Ionian Sea). So far published value (Kovačić & Šanda 2016) of intraspecific uncorrected p-distance for *G. niger* was based on three individuals and thus it was lower (2.0 %, ranging from 1.8 to 2.2 %) than the mean within species distance of cytochrome b found in this work (mean p distance 2.4 %). The values of mean intraspecific genetic divergence of the black goby are very high when compared with the species from the same lineage (*Gobius* lineage *sensu* Agorreta et al., 2013), where mean intraspecific genetic variability of cytochrome b varies from 0.13 % in *Gobius couchi* Miller & El-Tawil, 1974 (Kovačić & Šanda, 2016), to 5.1 % in *Zebrus pallaoroi* Kovačić, Šanda & Vukić, 2021 (Kovačić et al., 2021). The high intraspecific variability found in *Z. pallaoroi* is discussed as a possible sign of existence of two evolutionary lineages within that species (Kovačić et al., 2021).

The genetic distances between species within the *Gobius*-lineage (*sensu* Agorreta et al., 2013) are in general considerably high, ranging from 10 to 23 % (Kovačić et al., 2021) and the distatnces of the *G. niger* from the other species from the same clade varies from 18.0 % for *Gobius bucchichi* Steindachner, 1870 up to the 19.82 % for *Gobius fallax* Sarato, 1889 (Kovačić & Šanda, 2016).

5.2: Geographic structure

The analyses of two mitochondrial markers have revealed pronounced genetic structure dividing the population of the black goby into the four haplotype groups, “A”, “B”, “C” and

"D". The haplotype groups are not covered equally through the distribution range of the black goby, and their distribution shows marked geographic pattern (Fig. 8).

The haplotype group "A" has been found in the Atlantic Ocean, in the North Sea and in the western Mediterranean Sea. The haplotype group "B" is present in the individuals inhabiting the Adriatic Sea, and it is predominant haplotype group in the north Adriatic basin. The haplogroup "C" is the only haplotype group found in the Black Sea, and it is also present in the northern Adriatic Sea and in the western Mediterranean. The last haplotype group "D" is the only haplotype group in the Aegean and Ionian seas, and it also predominates in the southern Adriatic Sea. Moreover, four individuals bearing haplotypes belonging to this haplotype group were found also in the northern Adriatic Sea and one specimen in the western Mediterranean. The phylogenetic reconstruction of relationships between haplotype groups (Supplement VII and VIII) brought more questions, as the phylogenetic trees differ depending on the molecular marker, but due to the different length of sequence (1140 bp the cytochrome b, 296 bp the D loop) and shared mitochondrial origin of both markers, the phylogenetic tree reconstructed on the basis of the cytochrome b is expected to be more reliable.

Unfortunately, it was not possible to obtain samples from the southern and eastern part of the eastern Mediterranean, and thus the important part of information is missing to fully reconstruct the evolution of population structure within the Mediterranean Sea. However, the haplotype group "D" which predominates in the "central Mediterranean" area (the Aegean, Ionian and the southern Adriatic Seas) show pronounced differentiation from all the remaining haplotype groups (Tab. 11). On the other hand, the lowest mean molecular distance was found between the "B" (Adriatic) and "C" (Black Sea) haplotype groups (2.0 %). According to the phylogenetic reconstruction based on the cytochrome b, the haplotype group "D" presents the sister group to well supported monophyletic clade comprising the haplotype groups "A", "B" and "C". Unfortunately, the palaeontological record is scarce and insufficient for applicable calibration of molecular clocks, thus to calculate the exact timing of the vicariance events is not possible. As the separation of the common ancestor of the clades "A, B, C" from the basal group "D" was not dated, we are not able to properly allocate the first vicariance event in the time and space. The possible cause of the first vicariance event could be the separation of the population inhabiting the Aegean subbasin from the rest of the eastern Mediterranean. This would be possible as the consecutive sea level drops during Pleistocene (Dumitru et al., 2021) caused repeated partial closure of the Aegean Sea (Lambeck & Purcell, 2005; Simaiakis et al., 2017) which could have enhanced speciation. Actually, one specimen from the haplotype group "D" was found also in the western Mediterranean and two specimens in the northern Adriatic Sea

(Fig. 7), probably as a result of secondary contact of allopatric populations (Grant & Bowen, 1998, discussed later).

According to the phylogenetic reconstruction based on cytochrome b (see the Supplement VII), we can see that during the history, the monophyletic clade “A, B, C” experienced at least two independent vicariance or radiation events, dividing the clade into the three haplotype groups.

One possible event which could cause the vicariance of the population of the black goby was the division of Mediterranean Sea by the interruption of the narrow Sicilo-Tunisian Channel. This geological event was already described as important force causing vicariance of various marine fish taxa, for example the sea bass *Dicentrarchus labrax* Linnaeus, 1758 (Bahri-sfar et al., 2000), the mackerel *Scomber scombrus* Linnaeus, 1758 (Zardoya et al., 2004), or the sand goby *Pomatoschistus tortonesei* (Mejri et al., 2009), and also Giovannotti et al. (2009) described genetic partitioning of these basins for the population of *G. niger*.

From the results it is apparent that this barrier could influence the evolution of the population structure of the *G. niger*, but the presence of some haplotypes shared among regions (see the Fig. 7 and 15) testifies that the channel was not absolutely impenetrable. The level of permeability of the Sicilo-Tunisian Channel could have changed as a consequence of the climatic oscillations during the Pleistocene, when the sea level repeatedly fell down approximately 120 meters below the actual level (Bianchi et al., 2012; Dumitru et al., 2021). The sea level oscillations impacted greatly the area of the Sicilo-Tunisian Channel due to its low depth (Lambeck et al., 2004; Prampolini et al., 2017), and it is supposed to have been totally interrupted by land approximately 1.83 to 2.0 million years ago (Stöck et al., 2008). This can be in accordance with Giovannotti et al. (2009) who proposed the ancient expansion event of the population of *G. niger* approximately 1.3 million years ago. Thus, the temporal separation could have caused the allopatric cladogenesis of new lineage, which remained separated even after secondary re-opening of the Sicilo-Tunisian Channel, as described in the case of *Pomatoschistus marmoratus* (Mejri et al., 2011). The secondary contact of the two lineages which evolved in allopatry in the western Mediterranean Sea can be seen in the haplotype network (Fig. 7 and 11), and also it can be suggested from the diversity measures of both molecular markers, showing high both haplotype and nucleotide diversity (see the Tab. 10 for cytochrome b and the Tab. 37 for the D loop) (Grant & Bowen, 1998).

Under this scenario, the separation of the population by the Sicilo-Tunisian Channel would have caused the second vicariance event, dividing the western Mediterranean population (the haplotype group “A”) from the population inhabiting the south of the eastern

Mediterranean basin (the supposed ancestor of the haplotype group “C”). This hypothetical population could have been a source population colonizing also the Black Sea and the northern Adriatic Sea. In the case that this hypothesis is correct, the part of the western Mediterranean population which today belongs into the haplotype group “C” would be an imprint of the multiple secondary introduction of the eastern Mediterranean population within the western Mediterranean basin.

Indeed, this theory has weak points, among the others the high molecular diversity of the haplotype group “C” found in the western Mediterranean (0.74 %, ranging from 0.18 to 1.32 %, Tab. 24). If the actual representatives of this group present the secondary re-introduction of the original eastern Mediterranean population to the western Mediterranean basin, it would have had to happen multiple times independently, as there is no sign of founder effect. On the other hand, when compared the within group diversity of the haplotype groups found in the western Mediterranean samples (see the Table 17), the haplotype group “A” presents higher within group diversity (mean distance 0.83%, range from zero to 1.5 %, Tab. 24) than the group “C”, so it is possible to suggest that the haplotype group “C” was secondarily introduced to the western Mediterranean area which was already inhabited by the individuals bearing the haplotype group “A” (Provan & Bennett, 2008).

Very interesting is the presence of the haplotype group “C” (which is predominating in the western Mediterranean) in the Black Sea and in the northern Adriatic Sea. The population from the Black Sea shows signatures of the recent population expansion, as a high haplotype diversity, low nucleotide diversity and significant negative Tajima´s D (see the Tab. 30) (Tajima, 1989; Grant & Bowen, 1998). Also, when comparing the within region p-distances, the population from the Black Sea presents lowest mean genetic differentiation (0.2, ranging from zero to 0.97%) comparing with the mean genetic distance computed for the haplotype group “C” (0.69 %, range from zero to 1.4 %, Tab. 11) or with the western Mediterranean (see the Tab. 24). In comparison, the northern Adriatic part of the haplotype group “C” has a mean molecular diversity 0.63 % (range from zero to 0.88 %) (Tab. 29). Given the fact, that the presence of the haplotype group “C” was not detected in the “central Mediterranean” region, it is not possible to explain the introduction of this haplotype group into the Black Sea and into the northern Adriatic Sea without blending with the ”central Mediterranean” population.

The distribution of the haplotype group “B” is restricted to the area of the Adriatic Sea, where it presents a dominant haplotype group in the north, and one specimen from this haplotype group was found in the southern Adriatic Sea, where the haplotype group “D” predominates. According to the phylogenetic reconstruction based on the cytochrome b, the haplotype group “B” should be monophyletic sister clade do the group “A”. Interestingly, the highest molecular difference between the haplotype groups “D” and “B” (5.7 %) was found between the sample from the southern Adriatic Sea and northern Adriatic Sea, thus within the Adriatic subbasin.

5.3: Connectivity among regions and population history

As discussed in 5.2, the population is deeply divided into several haplotype groups and this division seems to have roots in repetitive allopatric events causing vicariance of lineages which was enhanced by the species limited mobility. The black goby is sedentary demersal species, and the dispersion of the species seems to occur mainly during the planktonic larval stage, which has mean duration of 28 days according to Planes (1998).The species interpopulation connectivity is supposed to be shaped by the oceanographic currents and fronts influencing the dispersing planktonic larvae (Galarza, et al., 2009; White et al., 2010) rather than by the lack of suitable habitats or prey (Kaschner et al*.,* 2019). In different parts of the area of distribution, the population underwent different evolutionary pathways due to the diverse climatic conditions and limitations in the species mobility.

The population from the area of the North-East Atlantic Ocean and the North Sea is moderately structured (global F_{ST} 0.061). The populations from the North Sea to the Iberian Peninsula presents only very low genetic differentiation, but the population from the Canary differ significantly ($F_{ST}>0.13$) from the rest of the Atlantic population. Similar pattern of reduction of population connectivity in the Macaronesian archipelagos was found also for *Hippocampus hippocampus* Linnaeus, 1758 (Woodall et al., 2011).

The Atlantic population of *G. niger* shows typical signature of fast demographic expansion, the star-shape of the haplotype network (Jenkins et al., 2018) supported with the significant negative values of the neutrality tests (see the Fig. 10 and the Tab. 16). Also, the recent expansion event can be suggested from the very low nucleotide diversity (0.00327 see the Tab. 16) together with high haplotype diversity (0.94) indicating rapid population growth enhancing the fixation of new mutations (Grant & Bowen, 1998). The population expansion probably occurred after the end of the last glacial maximum, when the ice sheet retreated and the sea level increased up to the actual level. During the last glacial maximum, the low sea level and the ice sheet covering the British islands (Hughes et al., 2016) forced the marine species into the lower latitudes where the reduced populations survived in so-called glacial refugia. The localization of the glacial refugia can be read from the molecular signature as described by Provan & Bennett (2008) who proposed that the possible glacial refugia should be expected on the locality with the highest actual molecular diversity. Such a place would be in the case of Atlantic population the Atlantic coast of Iberian Peninsula (see the Tab. 10 and the Tab. 17), which has much higher within-group genetic diversity (mean 0.46 %, Tab. 17) than the rest of the areas. Localization of glacial refugia on the coast of Iberian Peninsula was described also for some other marine taxa (Nieva et al., 2012; Francisco et al., 2014) including the sand gobies *Pomatoschistus minutus* and *P. microps* (Kettle et al., 2011).

The Atlantic Ocean is connected with the western Mediterranean through the narrow Strait of Gibraltar, where on the contact of the Atlantic and Mediterranean waters arises the Almeria-Oran front (see the 1.2.2), which is affecting connectivity of various benthic organisms (Patarnello et al., 2007). Interestingly, our results show that many of the haplotypes found in the western Mediterranean are very similar to the haplotypes found in the Atlantic Ocean, but only two haplotypes were shared among the Atlantic and Mediterranean individuals. The pairwise F_{ST}s revealed pronounced differentiation of the western Mediterranean population from the populations from the Atlantic Ocean, with the lowest F_{ST} value from Canary Islands (0.26). According to AMOVA between the regions of the Atlantic Ocean and the western Mediterranean (Tab. 20), the most of the genetic variance (79.6 %) is distributed within the populations, and the global F_{ST} (0.081) shows only moderate differentiation of the population. The western Mediterranean population itself shows no geographic structure (Fig. 11) and extremely high genetic variability, which is caused by the contact of three haplotype groups and also by the hyperdiversity of haplotype groups “A” and “C” (Tab. 10). The results indicate that the area of the Strait of Gibraltar is decreasing the population connectivity, but it does not present biogeographic break. The pattern of extremely high genetic variability of (mostly) singletons and limited number of shared haplotypes was already described in the case of *Gobius cruentatus* (Čekovská et al., 2020) or *Scorpaena maderensis* Valenciennes, 1833 (Francisco et al., 2021), According to (Francisco & Robalo, 2020; Francisco et al., 2021), this genetic pattern can occur once the successive self-recruitment becomes dominant over the larval drifting to further locations. The hyperdiversity of the mitochondrial DNA can also be a result of low sampling effort if the population was old enough to accumulate extreme genetic variability (Fourdrilis & Backeljau, 2019), or it can be produced by the augmented mutational rates of the molecular marker (Robalo et al., 2020).

Moreover, as there was found no geographic structure within the western Mediterranean and also no significant difference between the northern and southern western Mediterranean (Tab. 26), it can be suggested that the Balearic front (Barral et al., 2021) does not act as a barrier in the population of *Gobius niger*. The Balearic front was found to have moderate effect on the connectivity of two littoral fish species (*Diplodus vulgaris* Geoffroy Saint-Hilaire, 1817, *Oblada melanura* Linnaeus, 1758) and weak differentiation was found also among the populations of *Serranus cabrilla* Linnaeus, 1758 and *Tripterygion delaisi* Cadenat & Blache, 1970 (Galarza et al., 2009).

Even after reconnection of the western and eastern basin of the Mediterranean Sea, the Sicilo-Tunisian Channel acts as a strong barrier preventing gene exchange between the eastern and western basin for some marine taxa (Pascual et al., 2017). The current values of pairwise F_{STs} between geographic regions show extremely pronounced differentiation among eastern and western Mediterranean populations, with the values of pairwise F_{STs} between western Mediterranean and the eastern regions ranging from 0.45 for the northern Adriatic population up to the 0.84 for the "central Mediterranean" population (see the Tab. 15). The lower values of pairwise F_{STs} between the western Mediterranean and the northern Adriatic/ the Black Sea regions seems to be caused by the shared presence of haplotype group "C" which marks some genetic connection between those regions in the past, but it does not present signal of recent connectivity due to the big within group molecular distances. On the other hand, the presence of the "central Mediterranean" haplotypes in the specimens from the western Mediterranean region (one individual in the cytochrome b analysis, two individuals in the case of D loop, as there were more specimens analysed) indicates recent limited gene transfer from the eastern to the western Mediterranean. The Sicilo-Tunisian Channel does not act as a universal barrier for the marine biota. In the case of benthopelagic *Mugil cephalus* Linnaeus, 1758 (Durand et al., 2013), benthic *Scorpaena maderensis* (Francisco et al., 2021) or demersal *Hippocampus hippocampus* (Woodall et al., 2011), the populations are presenting homogeneous structure throughout Mediterranean Sea. No geographic discontinuity was also found within the populations of *Gobius cruentatus* and *Gobius geniporus* (Čekovská et al., 2020). On the other hand, the Sicilo-Tunisian Channel was found to be a strong barrier for sand gobies *Pomatoschistus marmoratus* (Mejri et al., 2011) or *Pomatoschistus tortonesei* (Mejri et al., 2009) but also for pelagic *Sprattus sprattus* (Debes et al., 2008). According to the Pascual et al. (2017), the Sicilo-Tunisian Channel in general cause the smallest reduction of the gene flow compared to the other barriers within the Mediterranean Sea.

The Aegean and Ionian populations seem to be strongly influenced by the complicated circulation patterns found within the subbasin (Hamad et al., 2006). The Etesian summer winds are strengthening the current flowing from the north of the Aegean Sea in the southward direction since beginning of summer until the early autumn (Hamad et al., 2006; Poupkou & Zanis, 2011). The timing of the Etesian winds strongly corelate with the reproductive period of the black goby (Bilgin & Onay, 2020) which can enhance the influence on the population structure. This one-way direction may be the cause of the moderate differentiation of the Aegean Sea population in relation to the Ionian and the Adriatic basins (see Tab. 35). The importance of the Ethesian winds and the strengthening of the Aegean current on the population connectivity was described also for the *Pomatoschistus marmoratus* (Seyhan-Ozturk & Engin, 2021). In the Ionian Sea, the strong stable quasi-circular anticyclonic front southwest of the Peloponnese (Hamad et al., 2006) is preventing the gene flow out of the area, which was described for various species, as for example for *Dicentrarchus labrax* (Bahri-sfar et al., 2000) or *Chromis chromis* Linnaeus, 1758 (Domingues et al., 2005).

The current driven by the general counter-clockwise circulation follows the Greek coast from Peloponnese peninsula in the north-west direction to the Adriatic sub-basin (Hamad et al., 2006) through the Otranto Strait. Although this narrow connection was considered migration barrier reducing the gene flow of benthic organisms (Pascual et al., 2017), the black goby seems to be influenced more by the complex circulation within the Adriatic sub-basin (Hariri, 2022), which acts as a barrier between the northern and southern Adriatic populations. The pairwise F_{ST} between the Ionian and the southern Adriatic seas finished with extremely low and nonsignificant value (the populations from the Ionian and southern Adriatic seas share four haplotypes), but between the southern and northern parts of the Adriatic Sea, it uncovered striking differentiation (0.487). The similar pattern of population divided by the within base circulation in the Adriatic Sea is well described for another benthic fish species *Tripterygion tripteronotum* Risso, 1810 (Sefc et al., 2020), which has similar ecology and life history as the black goby (Wirtz, 1978; Raventós & Macpherson, 2001).

The northern Adriatic population consists of three different haplotype groups “B”, “C” and “D” (see the Fig. 12), and the high value of both haplotype and nucleotide diversity (see the Tab. 27) are thus rather explained by the secondary contact of three allopatric lineages (Grant & Bowen 1998) than by stability of the population. The haplotype groups are not distributed equally, but the haplotype group “B” is dominating. All of the haplotypes found in the northern Adriatic region were unique for this geographic part, and most of the haplotypes were found as singletons. The presence of independent haplotype unit in the northern Adriatic Sea is consistent with the genetic structure described for the group of sand gobies (*sensu* McKay & Miller, 1997). (Tougard et al., 2021) is connecting the high diversity of the sand gobies with the climatic oscillations during the Pleistocene. The decline of the sea level has led to the disappearance of the marine environment in the northern part of the basin (Sikora et al., 2014), which could force the euryhaline species to survive in fluvio-lacustrine environment. The black goby exhibits also the tolerance to the low salinities (Paris & Quignard, 1971), which could have led to the similar pattern of isolation during the Pleistocene climatic oscillations and emerging of the haplotype group "B". This hypothesis can be supported by the distribution of the haplotypes of the haplogroup "B" indicating recent population expansion (Jenkins et al., 2018) and by the significant negative values of neutrality tests (see the Tab. 28).

Contrary to the southern part of the Adriatic Sea, the Black Sea basin was affected during the Pleistocene climatic oscillations by the disrupting of the connection with the Mediterranean Sea (Badertscher et al., 2011) resulting in the changes of the water salinity within the Black Sea basin (Aksu & Hiscott, 2022). The population of the black goby inhabiting the Black Sea show pronounced differentiation from all the other regions. Interestingly, the highest F_{ST} value (0.917) was found between the populations from the Black Sea and "central Mediterranean", which is geographically closest. The population from the Black Sea has quite low nucleotide diversity (0.00204) compared with other regions, and the haplotype network (Fig. 13) indicates population expansion, which is not fully supported by the tests of neutrality. Only the haplotype group "C" was found in the Black Sea, and all the haplotypes were private to this population. When compared with other regions where this haplotype group appeared (western Mediterranean and the north Adriatic Sea), the population from the Black Sea has lowest mean genetic variability (0.2% in the Black Sea, 0.6 % in the north Adriatic, 0.7 % in the western Mediterranean). From actual dataset, we are not able to distinguish, if the low diversity in the Black Sea is caused by the recent colonisation of the basin, or by the reduction of the population during the glacial phases. Episodic colonisation and isolation of the population in the Black Sea was described for *Scorpaena porcus* (Boissin et al., 2016) or *Hippocampus guttulatus* (Woodall et al., 2015), but in both cases this dynamic has led to the increase of genetic diversity. The structure found in the Black Sea population of *G. niger* has some similarities with the population structure of *Mugil cephalus* (Durand et al., 2013), as the Black Sea population of the *M. cephalus* shows increased frequency of the alleles which are in low frequency within Mediterranean Sea. However, none of the mentioned species has shown such a strict separation of the Black Sea population from the "central Mediterranean" region, as was found for the black goby. In the case of European Anchovy (*Engraulis encrasicolus*), Magoulas et al. (1996) have described origin of new haplotype group within the Black Sea basin, from where it was distributed throughout eastern Mediterranean. Given the geographic differences in the genetic variability of the haplotype group "C", the origin of this haplotype group within the Black Sea basin is rather not presumable.

5.4: Molecular markers

To investigate the genetic diversity and population structure of the black goby, the two mitochondrial markers, cytochrome b and D loop have been used. The crucial attribute of the mitochondrial DNA for the use as a good phylogeographic marker is the maternal inheritance lowering the effective population size (Brown, 2008), and high rate of evolution (Wilson et al., 1985). Given that characteristics, the mitochondrial DNA is accumulating the mutations within particular lineages, which enables to reconstruct the demographical and spatial histories of given lineages.

As the mitochondrial markers vary in length and in the mutation rate, they differ in the strength of the molecular signal of the past demographic changes (Brown et al., 1993; Lee et al., 1995). The sequences of D loop were only 296 base pairs long, and in the global population contained 36 segregation sites distinguishing 147 haplotypes. In comparison, the cytochrome b sequences were 1140 base pairs long, containing 269 segregation sites. In total, 290 haplotypes of cytochrome b were detected. Apparently, the longer and more variable cytochrome b was more suitable for finer scale depiction of population dynamics, albeit even the short D loop sequence has shown comparable results, and thus it is suitable for use in phylogeographic studies, where it can help to decrease expenses associated with the PCR preparation and sequencing.

To obtain more complex view on real population connectivity and gene flow, it would be useful to enrich the current analysis with the nuclear markers, as the biparental inheritance and higher effective population size of the nuclear genes (Larmuseau et al., 2010) and the possibility to uncover the hybridisation (Joly et al., 2009) can provide deeper information on the evolutionary history of the subpopulations of the black goby. For example, the combined use of mitochondrial and nuclear markers can reveal whether lineages observed on mitochondrial DNA separated by ancient events remained isolated, or if they reconnected together once they appeared in sympatry (Toews & Brelsford, 2012), and only the trace of the ancient isolation remained in their mitochondrial DNA.

6: Conclusions

In the current work, the genetic diversity of the populations of *Gobius niger* (Gobiidae, Gobiiformes) from numerous localities throughout its distribution range (north-east Atlantic and the Mediterranean and Black seas) was examined, using two mitochondrial markers, cytochrome b and D loop. The population of the black goby presents extremely high within species genetic variability, with the maximum uncorrected p-distance on cytochrome b of 6.1%, which is a very high value for fishes. The results revealed a complex structure of the population, dividing it into the four well defined haplotype groups, with unexpected geographic pattern and the highest mean genetic distance between them on cytochrome b of nearly 5.5% (maximum 6.1%). The high genetic differences between haplotype groups indicate their ancient origin. Geographically, while the broad region of the Atlantic distribution of the species is very homogeneous, with the most frequent central haplotype shared by most populations and the haplotypes from the whole region falling within a single haplotype group, the genetic structure of the populations within the Mediterranean Sea is very complex. The presence of haplotypes belonging to two or even three diversified haplotype groups was revealed at two localities in the western Mediterranean Sea and two localities in the Adriatic Sea, which can be attributed to a secondary contact of allopatric lineages.

The interconnection of the populations of the black goby from the Atlantic and the western Mediterranean Sea was revealed, the haplotypes predominating in the western Mediterranean Sea, belonged to the same haplotype group as the ones from the Atlantic population. This finding suggests that neither the Strait of Gibraltar, nor the Almeria-Oran front represents the barrier for the populations of the black goby. A very unusual and unexpected is the (past) connection of the western Mediterranean, Black Sea and the north Adriatic populations haplotypes, sharing one haplotype group. On the other hand, within the Adriatic Sea, two regions, each with a different haplotype group predominating, suggesting a very limited gene flow, apparently influenced by the circulation in the basin, was uncovered. Interestingly, the population from the southern Adriatic Sea had haplotypes belonging to the same haplotype group as the haplotypes of the populations from the Ionian and the Aegean seas. Additionally, multiple haplotypes were shared between south Adriatic and Ionian populations, which points to the fact than the Otranto Strait is not a barrier for gene flow between the populations of the black goby.

The results further suggest that the Sicilo-Tunisian Channel, as well as the Turkish strait system restrict the gene flow between the populations of the black goby.

In the Mediterranean Sea, the phylogeographic structure seems to be affected by the combination of paleoclimatic and past and actual hydrological patterns. The hydrology of the inhabited area appears to have crucial effect on the species connectivity, taking into consideration the demersal life style of adults and long pelagic larval duration of the black goby.

The current results are based on the mitochondrial data and thus elucidate the historical pattern conserved in maternally inherited lineages. On the other hand, the use of mitochondrial markers could mask recent phylogeographic structure, thus the addition of nuclear marker(s) is desirable to uncover the complete evolutionary history.

References

Agorreta, A., San Mauro, D., Schliewen, U., Tassell, J. L. Van, Kovačić, M., Zardoya, R., & Rüber, L. (2013). Molecular Phylogenetics and Evolution Molecular phylogenetics of Gobioidei and phylogenetic placement of European gobies. *Molecular Phylogenetics and Evolution*, *69*, 619–633.

Ahnelt, H., Bianco, P. G., & Schwammer, H. (1995). Systematics and zoogeography of *Knipowitschia caucaska* (Teleostei: Gobiidae) based on new records from the Aegean Anatolian area. *Ichthyol. Explor. Frshwaters*, *6*(1), 49–60.

Aksu, A. E., & Hiscott, R. N. (2022). Earth-science reviews persistent holocene outflow from the Black Sea to the eastern Mediterranean Sea still contradicts the Noah ' s flood hypothesis: A review of 1997 – 2021 evidence and a regional paleoceanographic synthesis for the latest Pleistocene-H. *Earth-Science Reviews*, *227*.

Arruda, L. M., Azevedo, J. N., & Neto, A. I. (1993). Abundance, age-structure and growth, and reproduction of Gobies (Pisces; Gobiidae) in the Ria de Aveiro Lagoon (Portugal). In *Estuarine, Coastal and Shelf Science*, *37*, 509–523.

Artegiani, A., Bregant, D., Paschini, E., Pinardi, N., Raicich, F., & Russo, A. (1997). The adriatic sea general circulation. Part II: Baroclinic circulation structure. *Journal of Physical Oceanography*, *27*(8), 1515–1532.

Badertscher, S., Fleitmann, D., Cheng, H., Edwards, R. L., Göktürk, O. M., & Zumbühl, A. (2011). Pleistocene water intrusions from the Mediterranean and Caspian seas into the Black Sea. *Nature Geoscience*, *4*, 236–239.

Bahri-sfar, L., Lemaire, C., & Hassine, O. K. Ben. (2000). Fragmentation of sea bass populations in the western and eastern Mediterranean as revealed by microsatellite polymorphism. *Proceedings of the Royal Society B: Biological Sciences*, *267*, 929–935.

Baker, A. J., & Marshall, H. D. (1997). Mitochondrial control region sequences as tools for understanding evolution. In *Avian molecular evolution and systematics*, 51–82.

Barral, Q., Zakardjian, B., Dumas, F., Garreau, P., Testor, P., & Beuvier, J. (2021). Characterization of fronts in the Western Mediterranean with a special focus on the North Balearic Front. *Progress in Oceanography*, *197*, 1–24.

Bell, J. D., & Harmelin-Vivien, M. (1983). Fish fauna of French Mediterranean Posidonia oceanica seagrass meadows. 2: feeding habits. *Tethys*, *11*(1), 1–14.

Bianchi, C. Nike, & Morri, C. (2000). Marine biodiversity of the Mediterranean Sea: Situation, problems and prospects for future research. *Marine Pollution Bulletin*, *40*(5), 367–376.

Bianchi, Carlo Nike, Morri, C., Chiantore, M., Montefalcone, M., Parravicini, V., & Rovere, A. (2012). Mediterranean Sea biodiversity between the legacy from the past and a future of change. In *Life in the Mediterranean Sea: A Look at Habitat Changes* (pp. 1–55).

Bilgin, S., & Onay, H. (2020). Seasonal growth and reproduction of the black goby*, Gobius niger* (Osteicgthyes: Perciformes: Gobiidae) in the southeastern Black Sea Region of Turkey. *Journal of Anatolian Environmental and Animal Sciences*, *5*(4), 466–474.

Boban, J., Isajlović, I., Zorica, B., Čikeš Keč, V., & Vrgoč, N. (2013). Biometry and distribution of the black goby *Gobius niger* (Linnaeus, 1758) in the adriatic sea. *Acta Adriatica*, *54*(2), 265–272.

Boissin, E., Micu, D., Janczyszyn-Le Goff, M., Neglia, V., Bat, L., Todorova, V., Panayotova, M., Kruschel, C., Macic, V., Milchakova, N., Keskin, Anastasopoulou, A., Nasto, I., Zane, L., & Planes, S. (2016). Contemporary genetic structure and postglacial demographic history of the black scorpionfish, *Scorpaena porcus*, in the Mediterranean and the Black Seas. *Molecular Ecology*, *25*(10), 2195–2209.

Boissin, Emilie, Hoareau, T. B., & Berrebi, P. (2011). Effects of current and historic habitat fragmentation on the genetic structure of the sand goby *Pomatoschistus minutus* (Osteichthys, Gobiidae). *Biological Journal of the Linnean Society*, *102*, 175–198.

Bonfardeci, A., Caruso, A., Bartolini, A., Bassinot, F., & Blanc-valleron, M. (2018). Distribution and ecology of the *Globigerinoides ruber — Globigerinoides elongatus* morphotypes in the Azores region during the late Pleistocene-Holocene. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *491*, 92–111.

Bouchereau, J. L., & Guelorget, O. (1998). Comparison of three Gobiidae (Teleostei) life history strategies over their geographical range. *Oceanologica Acta*, *21*(3), 503–517.

Brown, J. R., Beckenbach, A. T., & Smith, M. J. (1993). Intraspecific DNA sequence variation of the mitochondrial control region of white sturgeon (*Acipenser transmontanus*). *Molecular Biology and Evolution*, *10*(2), 326–341.

Brown, K. H. (2008). Fish mitochondrial genomics: sequence, inheritance and functional variation. *Journal of Fish Biology*, *72*, 355–374.

Carnevale, G., & Schwarzhans, W. (2022). Marine life in the Mediterranean during the messinian salinity crisis: a paleoichthyological perspective. *Rivista Italiana Di Paleontologia e Stratigrafia*, *128*(2), 283–324.

Casabianca, M. L., & Kiener, A. (1969). Gobiidés des étangs corses: systemátique, écologie, régime alimentaire et position dans les chaines trophiques. *Vie et Milieu*, *Observatoire Océanologique - Laboratoire Arago*, *1969*, 611–634.

Čekovská, K., Šanda, R., Eliášová, K., Kovačić, M., Zogaris, S., Pappalardo, A. M., Soukupová, T., & Vukić, J. (2020). Population genetic diversity of two marine gobies (Gobiiformes: Gobiidae) from the north-eastern Atlantic and the Mediterranean Sea. *Journal of Marine Science and Engineering*, *8*(792), 1–18.

Centurioni, L. R., & Niiler, P. P. (2003). On the surface currents of the Caribbean Sea. *Geophysical Research Letters*, *30*(6), 10–13.

Charria, G., Lazure, P., Le, B., Serpette, A., Reverdin, G., Louazel, S., Batifoulier, F., Dumas, F., Pichon, A., & Morel, Y. (2011). Surface layer circulation derived from Lagrangian drifters in the Bay of Biscay. *Journal of Marine Systems*, *109–110*, S60–S76.

Claridge, P. N., Hardisty, M. W., Potter, I. C., & Williams, C. V. (1985). Abundance, life history and ligulosis in the gobies (teleostei) of the inner severn estuary. *Journal of the Marine Biological Association of the United Kingdom*, *65*(4), 951–968.

Clement, M., D. Posada and K. A. Crandall 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology 9* (10): 1657-1660.

Como, S., van der Velde, G., & Magni, P. (2018). Temporal variation in the trophic levels of secondary consumers in a Mediterranean coastal lagoon (Cabras Lagoon, Italy). *Estuaries and Coasts*, *41*(1), 218–232.

Cowen, R. K., Lwiza, K. M. M., Sponaugle, S., Paris, C. B., & Olson, D. B. (2000). Connectivity of marine populations: Open or closed? *Science*, *287*(5454), 857–859.

Cowen, R. K., & Sponaugle, S. (2009). Larval dispersal and marine population connectivity. *Annual Review of Marine Science*, *1*, 443–466.

Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods 9*(8), 772.

Debes, P. V., Zachos, F. E., & Hanel, R. (2008). Mitochondrial phylogeography of the European sprat (*Sprattus sprattus* L ., Clupeidae) reveals isolated climatically vulnerable populations in the Mediterranean Sea and range expansion in the northeast Atlantic. *Molecular Ecology*, *17*, 3873–3888.

di Rago, J., Macadre, C., Lazowska, J., & Slonimski, P. P. (1993). The C-terminal domain of yeast cytochrome b is essential for a correct assembly of the mitochondrial cytochrome bc1 complex. *Federation of European Biochemical Societies Letters*, *328*(1, 2), 153–158.

Domingues, V., Bucciarelli, G., Almada, V. C., & Bernardi, G. (2005). Historical colonization and demography of the Mediterranean damselfish, *Chromis chromis*. *Molecular Ecology*, *14*, 4051–4063.

Doornbos, G., & Twisk, F. (1987). Density, growth and annual food consumption of gobiid fish in the saline Lake Grevelingen, The Netherlands. *Netherlands Journal of Sea Research*, *21*(1), 45–74.

Duggen, S., Hoernie, K., Van den Bogaard, P., Rüpke, L., & Morgan, J. P. (2003). Deep roots of the Messinian salinity crisis. *Nature*, *422*(10), 602–606.

Dumitru, O. A., Austermann, J., Polyak, V. J., Fornós, J. J., Asmerom, Y., Ginés, J., Ginés, A., & Onac, B. P. (2021). Sea-level stands from the Western Mediterranean over the past 6.5 million years. *Scientific Reports*, *11*(261), 1–10.

Durand, J. D., Blel, H., Shen, K. N., Koutrakis, E. T., & Guinand, B. (2013). Population genetic structure of *Mugil cephalus* in the Mediterranean and Black Seas: a single mitochondrial clade and many nuclear barriers. *Marine Ecology Progress Series*, *474*, 243–261.

Excoffier, L., and Lischer, H.E.L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.* 10, 564–567.

Fabi, G., & Giannetti, G. (1985). Growth parameters of the black goby (*Gobius niger* L.) in the Adriatic Sea, based on otholiths reading. *Rapp. Comm. Int. Mer Médit.*, *29*(8), 87–90.

Faillettaz, R., Blandin, A., Paris, C. B., Koubbi, P., & Irisson, J.-O. (2015). Sun-Compass Orientation in Mediterranean Fish Larvae. *PLoS ONE*, *10*(8), 1–15.

Fielding, S., Crisp, N., Allen, J. T., Hartman, M. C., Rabe, B., & Roe, H. S. J. (2001). Mesoscale subduction at the Almeria–Oran front Part 2. Biophysical interactions. *Journal of Marine Systems*, *30*, 287–304.

Filiz, H. (2013). Age, growth and feeding of the Black goby, *Gobius niger*, in Candarli Bay (Aegean Sea). *Rapp. Comm. Int. Mer Médit.*, *40*, 666.

Filiz, H., & Toğulga, M. (2009). Age and growth, reproduction and diet of the black goby, (*Gobius niger*) from Aegean Sea, Turkey. *Journal of FisheriesSciences.Com*, *3*(3), 243–265.

Firidin, S., Ozturk, R. C., Alemdag, M., Eroglu, O., Terzi, Y., Kutlu, I., Duzgunes, Z. D., Cakmak, E., & Aydin, I. (2020). Population genetic structure of turbot (*Scophthalmus maximus* L., 1758) in the Black Sea. *Journal of Fish Biology*, *97*, 1154–1164.

Fives, J. M. (1970). Investigations of the plankton of the west coast of Ireland: IV. Larval and post-larval stages of fishes taken from the plankton of the west coast in surveys during the years 1958-1966. *Proceedings of the Royal Irish Academy*, *70*(sect. B), 15–93.

Flores, J. A., Sierro, F. J., Francés, G., Vázquez , A., & Zamarreño , I. (1997). The last 100,000 years in the western Mediterranean: sea surface water and frontal dynamics as revealed by coccolithophores. In *Marine Micropaleontology 29*, 351-366.

Fourdrilis, S., & Backeljau, T. (2019). Highly polymorphic mitochondrial DNA and deceiving haplotypic differentiation: implications for assessing population genetic differentiation and connectivity. *BMC Evolutionary Biology*, *19*(92), 1–16.

Francisco, S. M., Almada, V. C., Faria, C., Velasco, E. M., & Robalo, J. I. (2014). Phylogeographic pattern and glacial refugia of a rocky shore species with limited dispersal capability: the case of Montagu's blenny (*Coryphoblennius galerita*, Blenniidae). *Marine Biology*, *161*(11), 2509–2520.

Francisco, Sara M, Castilho, R., Lima, C. S., Almada, F., Rodrigues, F., Radek, Š., Vukić, J., Pappalardo, A. M., Ferrito, V., & Robalo, J. I. (2021). Genetic hypervariability of a Northeastern Atlantic venomous rockfish. *PeerJ*, *9*, 1–21.

Francisco, Sara M, & Robalo, J. I. (2020). Time matters : genetic composition and evaluation of effective population size in temperate coastal fish species. *PeerJ*, *8*:e9098

Fries, B., Ekström, C. U., & Sundevall, C. (1892). *A history of Scandinavian fishes*, (2nd edition), P.A. Norsted & söner

Froese, R., & Pauly, D. (2022). *FishBase*. *8*, 2022.

Fu, Y. X., & Li, W. H. (1993). Statistical tests of neutrality of mutations. *Genetics*, *133*, 693–709.

Galarza, J. A., Carreras-Carbonell, J., Macpherson, E., Pascual, M., Roques, S., Turner, G. F., & Rico, C. (2009). The influence of oceanographic fronts and early-life-history traits on connectivity among littoral fish species. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(5), 1473–1478.

Garcia-Castellanos, D., Micallef, A., Estrada, F., Camerlenghi, A., Ercilla, G., Periáñez, R., & María, J. (2020). The Zanclean mega flood of the Mediterranean – Searching for independent evidence. *Earth-Science Reviews*, *201*, 103061.

Glez-Peña, D.; Gómez-Blanco, D.; Reboiro-Jato, M.; Fdez-Riverola F.; Posada D. (2010) ALTER: program-oriented format conversion of DNA and protein alignments. *Nucleic Acids Research*. Web Server issue. ISSN: 0305-1048

Gilg, M. R., & Hilbish, T. J. (2003). The geography of marine larval dispersal: coupling genetics with fine-scale physical oceanography. *Esology*, *84*(11), 2989–2998.

Giovannotti, M., La Mesa, M., & Caputo, V. (2009). Life style and genetic variation in teleosts: The case of pelagic (*Aphia minuta*) and benthic (*Gobius niger*) gobies (Perciformes: Gobiidae). *Marine Biology*, *156*(3), 239–252.

Grant, W. S., & Bowen, B. W. (1998). Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. *The Journal of Heredity*, *89*(8), 415–426.

Guindon S and Gascuel O (2003). A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Systematic Biology* 52: 696-704.

Gysels, E. S., Leentjes, V., & Volckaert, F. A. M. (2004). Small-scale clinal variation, genetic diversity and environmental heterogeneity in the marine gobies *Pomatoschistus minutus* and *P. lozanoi* (Gobiidae, Teleostei). *Heredity*, *93*, 208–214.

Habib, M., Lakra, W. S., Mohindra, V., Khare, P., Barman, A. S., Singh, A., Lal, K. K., Punia, P., & Khan, A. A. (2011). Evaluation of cytochrome b mtDNA sequences in genetic diversity studies of *Channa marulius* (Channidae: Perciformes). *Molecular Biology Reports*, *38*, 841–846.

Hajji, F., Ouannes-Ghorbel, A., Ghorbel, M., & Jarboui, O. (2013). Feeding habits of the black goby, *Gobius niger* (Linnaeus, 1758), in the Gulf of Gabes (Southern Tunisia). *Cahiers De Biologie Marine*, *54*, 133–141.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser*. *41*, 95-98.

Hamad, N., Millot, C., & Taupier-Letage, I. (2006). The surface circulation in the eastern basin ot the Mediterranean Sea. *Scientia Marina*, *70*(3), 457–503.

Hariri, S. (2022). Analysis of mixing structures in the Adriatic Sea using finite-size Lyapunov exponents. *Geophysical & Astrophysical Fluid Dynamics*, *116*(1), 20–37.

Hsü, K. J., Ryan, W. B. F., & Cita, M. B. (1973). Late miocene desiccation of the Mediterranean. *Nature*, *242*(March 23), 240–244.

Hughes, A. L. C., Gyllencreutz, R., Lohne, Ø. S., Mangerud, J. A. N., & Inge, J. (2016). The last Eurasian ice sheets – a chronological database and time-slice reconstruction, DATED-1. *An International Journal of Quaternary Research*, *January, 45*, 1–45.

Huyse, T., Van Houdt, J., & Volckaert, F. A. M. (2004). Paleoclimatic history and vicariant speciation in the ‘“sand goby”’ group (Gobiidae, Teleostei). *Molecular Phylogenetics and Evolution*, *32*, 324–336.

Iglesias, J. (1981). Spatial and temporal changes in the demersal fish community of the Ria de Arosa (NW Spain). *Marine Biology*, *65*(2), 199–208.

Immler, S., Mazzoldi, C., & Rasotto, M. B. (2004). From sneaker to parental male: change of reproductive traits in the black goby, *Gobius niger* (Teleostei, Gobiidae). *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, *301*, 177–185.

Jenkins, T. L., Castilho, R., & Stevens, J. R. (2018). Meta-analysis of northeast Atlantic marine taxa shows contrasting phylogeographic patterns following post-LGM expansions. *PeerJ*, *6*, 1–23.

Joly, S., McLenachan, P. A., & Lockhart, P. J. (2009). A statistical approach for distinguishing hybridization and incomplete lineage sorting. *American Naturalist*, *174*(2), E54–E70.

Joyeux, J.-C., Bouchereau, J.-L., & Tomasini, J.-A. (1991). La reproduction de *Gobius niger* (Pisces, Gobiidae) dans la lagune de Maguio - France. *Vie et Milieu*, *41*(2/3), 97–106.

Joyeux, J.-C., Tomasini, J.-A., & Bouchereau, J.-L. (1991). Le régime alimentaire de *Gobius niger* Linné, 1758 (Teleostei, Gobiidae) dans la lagune de Mauguio — France. *Annales Des Sciences Naturelles. Zoologie et Biologie Animale*, *12*(2), 57–69.

Kalimeris, A., & Kassis, D. (2020). Sea surface circulation variability in the Ionian-Adriatic Seas. *Progress in Oceanography*, *189*.

Kalkan, E., Kurtuluş, A., Maraci, Ö., & Bilgin, R. (2011). Is the Bosphorus Strait a barrier to gene flow for the Mediterranean mussel, *Mytilus galloprovincialis* (Lamarck, 1819)? *Marine Biology Research*, *7*, 690–700.

Kara, M. H., & Quignard, J.-P. (2019). *Fishes in Lagoons and Estuaries in the Mediterranean 2*. ISTE Ltd, Wiley & Sons, Inc.

Kaschner, K., K. Kesner-Reyes, C. Garilao, J. Rius-Barile, T. Rees, and R. Froese. 2019. AquaMaps: Predicted range maps for aquatic species. *World wide web electronic publication*, www.aquamaps.org, version 10/2019.

Kettle, A. J., Morales-Muñiz, A., Roselló-Izquierdo, E., Heinrich, D., & Vøllestad, L. A. (2011). Refugia of marine fish in the northeast Atlantic during the last glacial maximum: Concordant assessment from archaeozoology and palaeotemperature reconstructions. *Climate of the Past*, *7*(1), 181–201.

Klein, B., & Siedler, G. (1989). On the Origin of the Azores Current. *Journal of Geophysical Research*, *94*(C5), 6159–6168.

Kovačić, M. & Patzner, R.A. (2011). North-Eastern Atlantic and Mediterranean Gobies. *The Biology of Gobies*, Patzner, R.A., Van Tassell, J.L., Kovačić, M. & Kapoor, B.G., (eds.), *Science Publishers Inc., Enfield*, pp. 177-206.

Kovačić, M., & Šanda, R. (2016). A new species of *Gobius* (Perciformes: Gobiidae) from the Mediterranean Sea and the redescription of *Gobius bucchichi*. *Journal of Fish Biology*, *88*(3), 1104–1124.

Kovačić, M., Šanda, R., Čekovská, K., Soukupová, T., & Vukić, J. (2021). *Zebrus pallaoroi* sp.

nov.: a new species of goby (Actinopterygii: Gobiidae) from the Mediterranean Sea with a dna-based phylogenetic analysis of the *Gobius*-lineage. *Contributions to Zoology*, *90*, 285–317.

Kruschel, C., & Schultz, S. T. (2011). Juvenile *Gobius niger* avoids seagrass in the presence and uncertain absence of seagrass-inhabiting predators. *Journal of Experimental Marine Biology and Ecology*, *409*(1–2), 240–246.

Labropoulou, M., & Papadopoulou-Smith, K.-N. (1999). Foraging behaviour patterns of four sympatric demersal fishes. *Estuarine, Coastal and Shelf Science*, *49*, 99–108.

Labropoulou, Mary, & Markakis, G. (1998). Morphological-dietary relationships within two assemblages of marine demersal fishes. *Environmental Biology of Fishes*, *51*(3), 309–319.

Lacroix, G., Maes, G. E., Bolle, L. J., & Volckaert, F. A. M. (2012). Modelling dispersal dynamics of the early life stages of a marine flatfish (*Solea solea* L .). *Journal of Sea Research*.

Lambeck, K., Antonioli, F., Purcell, A., & Silenzi, S. (2004). Sea-level change along the Italian coast for the past 10,000 yr. *Quaternary Science Reviews*, *23*(14–15), 1567–1598.

Lambeck, K., & Purcell, A. (2005). Sea-level change in the Mediterranean Sea since the LGM: Model predictions for tectonically stable areas. *Quaternary Science Reviews*, *24*(18–19), 1969–1988.

Larmuseau, M. H. D., Raeymaekers, J. A. M., Hellemans, B., Van Houdt, J. K. J., & Volckaert, F. A. M. (2010). Mito-nuclear discordance in the degree of population differentiation in a marine goby. *Heredity*, *105*, 532–542.

Lee, W., Conroy, J., Howell, W. H., & Kocher, T. D. (1995). Structure and evolution of teleost mitochondrial control regions. *Journal of Molecular Evolution*, *41*, 54–66.

Leis, J. M., & Carson-Ewart, B. M. (2000). Behaviour of pelagic larvae of four coral-reef fish species in the ocean and an atoll lagoon. *Coral Reefs*, *19*, 247–257.

Leis, J. M., Hay, A. C., & Trnski, T. (2006). In situ ontogeny of behaviour in pelagic larvae of three temperate, marine, demersal fishes. *Marine Biology*, *148*(3), 655–669.

Letourneur, Y., Darnaude, A., Salen-Picard, C., & Harmelin-Vivien, M. (2001). Spatial and temporal variations of fish assemblages in a shallow Mediterranean soft-bottom area (Gulf of Fos, France). *Oceanologica Acta*, *24*(3), 273–285.

Locatello, L., Santon, M., Mazzoldi, C., & Rasotto, M. B. (2017). The marbled goby, *Pomatoschistus marmoratus*, as a promising species for experimental evolution studies. *Organisms, Diversity, Evolution*, *17*, 709–716.

Machordom, A., & Doadrio, I. (2001). Evidence of a cenozoic Betic-Kabilian connection based on freshwater fish phylogeography (*Luciobarbus*, cyprinidae). *Molecular Phylogenetics and Evolution*, *18*(2), 252–263.

Magnhagen, C. (1988). Changes in foraging as a response to predation risk in two gobiid fish species, *Pomatoschistus minutus* and *Gobius niger*. *Marine Ecology Progress Series*, *49*(1), 21–26.

Magoulas, A., Tsimenides, N., & Zouros, E. (1996). Mitochondrial DNA phylogeny and the reconstruction of the population history of a species: The case of the European anchovy (*Engraulis encrasicolus*). *Molecular Biology and Evolution*, *13*(1), 178–190.

Malavasi, S., Franco, A., Fiorin, R., Franzoi, P., Torricelli, P., & Mainardi, D. (2005). The shallow water gobiid assemblage of the Venice Lagoon: Abundance, seasonal variation and habitat partitioning. *Journal of Fish Biology*, *67*(SUPPL. B), 146–165.

Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Research*, *27*(1), 209–220.

Massie, R., Wong, L.-J., & Milone, M. (2010). Excercise intolerance due to cytochrome b mutation. *MUSCLE & NERVE*, *42*(July), 136–140.

Mazzoldi, C., & Rasotto, M. B. (2002). Alternative male mating tactics in *Gobius niger*. *Journal of Fish Biology*, *61*(1), 157–172.

McKay, S. I., & Miller, P. J. (1997). The affinities of european sand gobies (Teleostei: Gobiidae). *Journal of Natural History*, *31*(10), 1457–1482.

Mejri, R., Arculeo, M., Hassine, O. K. Ben, & Lo Brutto, S. (2011). Molecular Phylogenetics and Evolution Genetic architecture of the marbled goby *Pomatoschistus marmoratus* (Perciformes, Gobiidae) in the Mediterranean Sea. *Molecular Phylogenetics and Evolution*, *58*, 395–403.

Mejri, R., Lo Brutto, S., Hassine, O. K. Ben, & Arculeo, M. (2009). A study on *Pomatoschistus tortonesei* Miller 1968 (Perciformes, Gobiidae) reveals the Siculo-Tunisian Strait (STS) as a breakpoint to gene flow in the Mediterranean basin. *Molecular Phylogenetics and Evolution*, *53*, 596–601.

Miller, P.J., 1986. Gobiidae. p. 1019-1085. In P.J.P. Whitehead, M.-L. Bauchot, J.-C. Hureau, J. Nielsen and E. Tortonese (eds.) Fishes of the North-eastern Atlantic and the Mediterranean. Volume 3. UNESCO, Paris.

Miller, M.A., Pfeiffer, W., and Schwartz, T. (2010) "Creating the CIPRES Science Gateway for inference of large phylogenetic trees" in *Proceedings of the Gateway Computing Environments Workshop (GCE)*, 14 Nov. 2010, New Orleans, LA pp 1 - 8.

Millot, C. (1999). Circulation in the Western Mediterranean Sea. In *Journal of Marine Systems, 20*(1999), 423-442.

Millot, C., & Taupier-Letage, I. (2005). Circulation in the Mediterranean Sea. *Hdb Env Chem*, *5*(June), 29–66.

Nash, R. D. M. (1984). Aspects of the biology of the black goby, *Gobius niger* L., in Oslofjorden, Norway. *Sarsia*, *06*(29), 55–61.

Nickols, K. J., Wilson White, J., Largier, J. L., & Gaylord, B. (2015). Marine population connectivity: Reconciling large-scale dispersal and high self-retention. *The American Naturalist, 185*(2), 196–211.

Nieto, P., & Alberto, L. J. (1993). Gobiidae y Bleniidae de la zona intermareal de la costa suroccidental de Andalucía (España). *Misc. Zool.*, *17*, 179–188.

Nieva, J., Pearson, G. A., Valero, M., & Serrao, E. (2012). Drifting fronds and drifting alleles: range dynamics, local dispersal and habitat isolation shape the population structure of the estuarine seaweed *Fucus ceranoides*. *Journal of Biogeography*, *39*, 1167–1178.

Olson, D. B., Kourafalou, V. H., Johns, W. E., Samuels, G., & Veneziani, M. (2007). Aegean surface circulation from a satellite-tracked drifter array. *Journal of Physical Oceanography*, *37*, 1898–1917.

Ostellari, L., Bargelloni, L., Penzo, E., Patarnello, P., & Patarnello, T. (1996). Optimization of single-strand conformation polymorphism and sequence analysis of the mitochondrial control region in *Pagellus bogaraveo* (Sparidae, Teleostei): rationalized tools in fish population biology. *Animal Genetics*, *27*, 423–427.

Palumbii, S. R. (2003). Population Genetics , Demographic Connectivity, and the Design of Marine Reserves, *Ecological Applications, 13*(1) *,* S14-13.

Pampoulie, C., Chauvelon, P., Rosecchi, E., Bouchereau, J. L., & Crivelli, A. J. (2001). Environmental factors influencing the gobiid assemblage of a Mediterranean lagoon: Empirical evidence from a long-term study. *Hydrobiologia*, *445*, 175–181.

Pampoulie, C., Priour, F., Bouchereau, J. L., Rosecchi, E., & Crivelli, A. J. (1999). Reproductive traits of *Gobius niger* (Teleostei: Pisces) following a salinity stress: Is it really a sedentary lagoon species? *Journal of the Marine Biological Association of the United Kingdom*, *79*(5), 961–962.

Pampoulie, Christophe, Rosecchi, E., Bouchereau, J. L., & Crivelli, A. J. (1999). Life history traits of *Pomatoschistus minutus* in the Rhône Delta, France. *Journal of Fish Biology*, *55*, 892–896.

Paris, J., & Quignard, J. P. (1971). La faune ichtiologique des étangs languedociens de sete a carnon. *Vie et Milieu*, *Supplement*, 301–327.

Pascual, M., Rives, B., Schunter, C., & MaCpherson, E. (2017). Impact of life history traits on gene flow: A multispecies systematic review across oceanographic barriers in the Mediterranean Sea. *PLoS ONE*, *12*(5), 1–20.

Patarnello, T., Volckaert, F. A. M. J., & Castilho, R. (2007). Pillars of Hercules: is the Atlantic–Mediterranean transition a phylogeographical break? *Molecular Ecology*, *16*, 4426–4444.

Patzner R.A., Van Tassell J.L., Kovačić M. & Kapoor B.G. (2011) The biology of gobies. CRC Press, *Science Publishers*, Taylor & Francis Group, New York.

Peakall, R. and Smouse P.E. (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*. 6, 288-295.

Peakall, R. and Smouse P.E. (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28, 2537-2539.

Peliz, Á., Dubert, J., Santos, A. M. P., Oliveira, P. B., & Le Cann, B. (2005). Winter upper ocean circulation in the western Iberian basin—fronts, eddies and poleward flows: an overview. *Deep-Seaa Research I*, *52*, 621–646.

Pérez-Ruzafa, A., García-Charton, J. A., Barcala, E., & Marcos, C. (2006). Changes in benthic fish assemblages as a consequence of coastal works in a coastal lagoon: The Mar Menor (Spain, Western Mediterranean). *Marine Pollution Bulletin*, *53*(1), 107–120.

Petersen, C.G.J. (1919). Our gobies (Gobiidae). From the egg to the adult stages. *Reports of the Danish Biological Station*, 26:45-66.

Pinot, J.-M., López-Jurado, J. L., & Riera, M. (2002). The CANALES experiment (1996-1998). Interannual, seasonal, and mesoscale variability of the circulation in the Balearic Channels. *Progress in Oceanography*, *55*, 335–370.

Planes, S. (1998). Genetic diversity and dispersal capabilities in marine fish. *Evolutionary Biology*, 30, 253-198.

Poupkou, A., & Zanis, P. (2011*).* Present climate trend analysis of the Etesian winds in the Aegean Sea. *Theor Appl Climatol, 106*, 459–472.

Prampolini, M., Foglini, F., Biolchi, S., Devoto, S., Angelini, S., & Soldati, M. (2017). Geomorphological mapping of terrestrial and marine areas, northern Malta and Comino (central Mediterranean Sea). *Journal of Maps*, *13*(2), 457–469.

Provan, J., & Bennett, K. D. (2008). Phylogeographic insights into cryptic glacial refugia. *Trends in Ecology and Evolution*, *23*(10), 564–571.

Quispe-Becerra, J. I. (2014). Dinámica espacio-temporal del ictioplancton del Mar Menor (SE de España) y factores ambientales asociados. In *Universidad de Murcia*.

Rabineau, M., Berné, S., Olivet, J., Aslanian, D., Guillocheau, F., & Joseph, P. (2006). Paleo sea levels reconsidered from direct observation of paleoshoreline position during Glacial Maxima (for the last 500, 000 years). *Earth and Planetary Science Letters*, *252*(1–2), 119–137.

Rambaut, A., Drummond, A.J., Xie, D., Baele, G., and Suchard, M.A. (2018). Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. *Syst. Biol*. 00, 1–3.

Rasotto, M. B., & Mazzoldi, C. (2002). Male traits associated with alternative reproductive tactics in *Gobius niger*. *Journal of Fish Biology*, *61*(1), 173–184.

Raventós, N., & Macpherson, E. (2001). Planktonic larval duration and settlement marks on the otoliths of Mediterranean littoral fishes. *Marine Biology*, *138*, 1115–1120.

Relvas, P., Barton, E. D., Dubert, J., Oliveira, P. B., Peliz, Á., da Silva, J. C. B., & Santos, A. M. P. (2007). Physical oceanography of the western Iberia ecosystem: Latest views and challenges. *Progress in Oceanography*, *74*, 149–173.

Robalo, J. I., Francisco, S. M., Vendrell, C., Lima, C. S., Pereira, A., Brunner, B. P., Dia, M., Gordo, L., & Castilho, R. (2020). Against all odds: a tale of marine range expansion with maintenance of extremely high genetic diversity. *Scientific Reports*, *10*(1).

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins S.E., Sánchez-Gracia, A. (2017) DnaSP v6: DNA Sequence Polymorphism Analysis of Large Datasets *Mol. Biol. Evol.* 34: 3299-3302

Rüber, L., & Agorreta, A. (2012). Molecular systematics of Gobioid fishes. In *The Biology of Gobies* (Issue September 2011, pp. 23–50).

Şalcıoğlu, A., Gubili, C., Krey, G., Sönmez, A. Y., & Bilgin, R. (2020). Phylogeography and population dynamics of the Eastern Mediterranean whiting (*Merlangius merlangus*) from the Black Sea, the Turkish Straits System, and the North Aegean Sea. *Fisheries Research*, *229*, 105614

Santos AM, Cabezas MP, Tavares AI, Xavier R, Branco M (2016) tcsBU: a tool to extend TCS network layout and visualization. *Bioinformatics*32(4), 627-8.

Scaggiante, M., Rasotto, M. B., Romualdi, C., & Pilastro, A. (2005). Territorial male gobies respond aggressively to sneakers but do not adjust their sperm expenditure. *Behavioral Ecology*, *16*(6), 1001–1007.

Schwarzhans, W., Agiadi, K., & Carnevale, G. (2020). Late miocene–early pliocene evolution of mediterranean gobies and their environmental and biogeographic significance. *Rivista Italiana Di Paleontologia e Stratigrafia*, *126*(3), 657–724.

Sefc, K. M., Wagner, M., Zangl, L., Weiß, S., Steinwender, B., Arminger, P., Weinmaier, T., Balkic, N., Kohler, T., Inthal, S., Kovačić, M., Zogaris, S., Hahn, C., & Koblmüller, S. (2020). Phylogeographic structure and population connectivity of a small benthic fish (*Tripterygion tripteronotum*) in the Adriatic Sea. *Journal of Biogeography*, *47*(June), 2502–2517.

Selkoe, K A, & Toonen, R. J. (2011). Marine connectivity: a new look at pelagic larval duration and genetic metrics of dispersal. *Marine Ecology Progress Series*, *436*, 291–305.

Selkoe, Kimberly A., Aloia, C. C. D., Crandall, E. D., Iacchei, M., Liggins, L., Puritz, J. B., Vo Der Heyden, S., & Toonen, R. J. (2016). A decade of seascape genetics: contributions to basic and applied marine connectivity. *Marine Ecology Progress Series*, *554*, 1–19.

Selkoe, Kimberly A, Henzler, C. M., & Gaines, S. D. (2008). Seascape genetics and the spatial ecology of marine populations. *Fish and Fisheries*, *9*(4), 363–377.

Serrano Gordo, L., & Nogueira Cabral, H. (2001). The fish assemblage structure of a hydrologically altered coastal lagoon: The Óbidos lagoon (Portugal). *Hydrobiologia*, *459*, 125–133.

Seyhan-Ozturk, D., & Engin, S. (2021). Genetic diversity of marbled goby populations in the Anatolian coasts of the north-eastern Mediterranean. *Journal of the Marine Biological Association of the United Kingdom*, *101*, 419–429.

Shanks, A. L. (2009). Pelagic larval duration and dispersal distance revisited. *Biological Bulletin*, *216*(3), 373–385.

Siegel, D. A., Kinlan, B. P., Gaylord, B., & Gaines, S. D. (2003). Lagrangian descriptions of marine larval dispersion. *Marine Ecology Progress Series*, *260*, 83–96.

Sikora, M., Mihanović, H., & Vilibić, I. (2014). Paleo-coastline of the Central Eastern Adriatic Sea, and paleo-channels of the cetina and neretva rivers during the last glacial maximum. *Acta Adriatica*, *55*(1), 3–18.

Simaiakis, S. M., Rijsdijk, K. F., Koene, E. F. M., Norder, S. J., Boxel, J. H. Van, Stocchi, P., Hammoud, C., Kougioumoutzis, K., Georgopoulou, E., Loon, E. Van, Tjørve, K. M. C., & Tjørve, E. (2017). Geographic changes in the Aegean Sea since the Last Glacial Maximum: Postulating biogeographic effects of sea-level rise on islands. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *471*, 108–119.

Stamatakis: "RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies". In *Bioinformatics*, 2014, open access link: http://bioinformatics.oxfordjournals.org/content/early/2014/01/21/bioinformatics.btu033.abstract? keytype=ref&ijkey=VTEqgUJYCDcf0kP

Stefanni, S., & Thorley, J. L. (2003). Mitochondrial DNA phylogeography reveals the existence of an Evolutionarily Significant Unit of the sand goby *Pomatoschistus minutus* in the Adriatic (Eastern Mediterranean). *Molecular Phylogenetics and Evolution*, *28*(3), 601–609.

Stergiou, K. I., & Karpouzi, V. S. (2002). Feeding habits and trophic levels of Mediterranean fish. In *Reviews in Fish Biology and Fisheries*, 11(3), 217-254

Stöck, M., Sicilia, A., Belfiore, N. M., Buckley, D., Brutto, S. Lo, Valvo, M. Lo, & Arculeo, M. (2008). Post-Messinian evolutionary relationships across the Sicilian channel: Mitochondrial and nuclear markers link a new green toad from Sicily to African relatives. *BMC Evolutionary Biology*, *8*(56), 1–19.

Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ & Rambaut A (2018) Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10 *Virus Evolution 4*, vey016.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, *123*, 585–595.

Tamura K, Stecher G, and Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Molecular Biology and Evolution 38*, 3022-3027.

Technelysium Pty Ltd. Chromas, version 2.6.6. http://technelysium.com.au/wp/chromas.html

Thacker, C. E. (2015). Biogeography of goby lineages (Gobiiformes: Gobioidei): origin, invasions and extinction throughout the Cenozoic. *Journal of Biogeography*, *42*, 1615–1625.

Thacker, C. E., Gkenas, C., Triantafyllidis, A., Malavasi, S., & Leonardos, I. (2019). Phylogeny, systematics and biogeography of the European sand gobies (Gobiiformes: Gobionellidae). In *Zoological Journal of the Linnean Society, 185,* 212-225.

Toews, D. P. L., & Brelsford, A. (2012). The biogeography of mitochondrial and nuclear discordance in animals. *Molecular Ecology*, *21*(16), 3907–3930.

Tougard, C., Folly, J., & Berrebi, P. (2014). New light on the evolutionary history of the common goby (*Pomatoschistus microps*) with an emphasis on colonization processes in the Mediterranean Sea. *PLoS ONE*, *9*(3), 1–12.

Tougard, C., Vukić, J., Ahnelt, H., Buj, I., Kovačić, M., Moro, G. A., Tutman, P., & Šanda, R. (2021). Quaternary climatic cycles promoted (re)colonization and diversification events in Adriatic sand gobies. *Journal of Zoological Systematics and Evolutionary Research*, *00*, 1–16.

Turan, C., Ozturk, B., Caliskan, M., Duzgunes, E., Gurlek, M., Yaglioglu, D., Hazar, D., & Sevenler, S. (2009). Genetic variation of Atlantic horse mackerel (*Trachurus trachurus*) in the Turkish waters. *Cah. Biol. Mar*, *50*, 207–213.

Turrell, W. R. (1992). New hypotheses concerning the circulation of the northern North Sea and its relation to North Sea fish stock recruitment. *Journal of Marine Science*, *49*, 107–123.

Vaas, K. F., Vlasblom, A. G., & De Koeijer, P. (1975). Studies on the Black goby (*Gobius niger*, Gobiidae, Pisces) in the Veerse Meer, SW Netherlands. *Netherlands Journal of Sea Research*, *9*(1), 56–68.

Vesey, G., & Langford, T. E. (1985). The biology of the black goby, *Gobius niger* L., in an English south-coast bay. *Journal of Fish Biology*, *27*, 417–429.

Voytas, D. (2000). Agarose gel electrophoresis of wheat starch. *Current Protocols in Molecular Biology*, *Supplement 51*, 2.5A.3-2.5A.9.

Warner, R. R., & Hughes, T. P. (1988). The population dynamics of reef fishes. *Proceeding of the 6th Internation Coral Reef Symposium*, *1*, 149–155.

White, C., Selkoe, K. A., Watson, J., Siegel, D. A., Zacherl, D. C., & Toonen, R. J. (2010). Ocean currents help explain population genetic structure. *Proceedings of the Royal Society B: Biological Sciences*, *277*(1688), 1685–1694.

Wilson, A. C., Cann, R. L., Carrii, S. M., George, M., Gyllenstenis, U. L. F. B., Kathleen, M., Higuchi, R. G., Stephen, R., Prager, E. M., Sage, R. D., & Stoneking, M. (1985). Mitochondrial DNA and two perspectives on evolutionary genetics. *Biological Journal of the Linnean Society*, *26*, 375–400.

Wirtz, P. (1978). The behaviour of the Mediterranean *Ttipterygion* species (Pisces, Blennioidei). *Z. Tierpsychol.*, *48*, 142–174.

Woodall, L. C., Koldewey, H. J., Boehm, J. T., & Shaw, P. W. (2015). Past and present drivers of population structure in a small coastal fish , the European long snouted seahorse *Hippocampus guttulatus*. *Conservation Genetics*, *16*(5), 1139–1153.

Woodall, L. C., Koldewey, H. J., & Shaw, P. W. (2011). Historical and contemporary population genetic connectivity of the European short-snouted seahorse *Hippocampus hippocampus* and implications for management. *Journal of Fish Biology*, *78*, 1738–1756.

Woodson, C. B., McManus, M. A., Tyburczy, J. A., Barth, J. A., Washburn, L., Caselle, J. E., Carr, M. H., Malone, D. P., Raimondi, P. T., Menge, B. A., & Palumbi, S. R. (2012). Coastal fronts set recruitment and connectivity patterns across multiple taxa. *Limnology and Oceanography*, *57*(2), 582–596.

Zaitsev, Y. (2008). *Introduction to the Black Sea Ecology* (1st edition). Smil Editing and Publishing Agency ltd.

Zardoya, R., Castilho, R., Grande, C., Favre-Krey, L., Caetano, S., Marcato, S., Krey, G., & Patarnello, T. (2004). Differential population structuring of two closely related fish species, the mackerel (*Scomber scombrus*) and the chub mackerel (*Scomber japonicus*), in the Mediterranean Sea. *Molecular Ecology*, *13*, 1785–1798.

Zarokanellos, N. D., Rudnick, D. L., Garcia-jove, M., Mourre, B., Ruiz, S., Pascual, A., & Tintoré, J. (2022). Frontal Dynamics in the Alboran Sea: 1. Coherent 3D Pathways at the Almeria-Oran Front Using Underwater Glider Observations. *Journal of Geophysical Research: Oceans*, *127*, 1–24.

Zenkevitch, L. (1963). *Biology of the Seas of the U.S.S.R.,* New York, Interscience Publ.